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Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine also produces relatively selective damage to nigrostriatal dopamine neurons and is a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease are unknown.

Several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, may account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals and excitatory amino acids predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. *The major objective is to examine the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity and bioenergetic and oxidative stress to produce damage to nigrostriatal dopamine neurons.* A multidisciplinary approach will be used as well as pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

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INTRODUCTION

Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine, also produces relatively selective damage to nigrostriatal dopamine neurons and is rapidly becoming a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease have escaped definition.

We propose that several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals, excitatory amino acids, and alter energy metabolism, predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. *The major objective is to examine in rats the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity, bioenergetic stress, and oxidative stress to produce damage to nigrostriatal dopamine neurons.* A multidisciplinary approach of *in vivo* and *in vitro* biochemical and histochemical methods will be used. In addition, pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

ANNUAL PROGRESS REPORT

OBJECTIVE 1:
To examine the interactions between methamphetamine, environmental stress and excitotoxicity

Results:
Ten days of unpredictable stress 1) augmented the acute increase in extracellular striatal dopamine concentrations in response to injections of 7.5 or 10 mg/kg METH and 2) produced greater depletions of striatal dopamine 7 days following the injection regimen and 3) exacerbated the METH-induced hyperthermia. These studies have been accepted for publication in the journal *Neuroscience* 124: 637-46, 2004. The manuscript is enclosed.

Discussion:
Several mechanisms may contribute to the potentiated decreases in dopamine tissue content and the acute increases in hyperthermia or extracellular dopamine in the striatum. The precise effects of repeated, unpredictable stress on the brain

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are unknown but alterations in 5-HT receptors, the dopaminergic system, or excitatory amino acid transmission may account for the enhanced hyperthermia, mortality, extracellular dopamine concentrations, or depletions of dopamine content in the striatum. Due to the broad overlap of stress and drug use, the increased vulnerability of the brain by exposure to unpredictable stressors may be important for understanding the potential detrimental effects of drugs of abuse as well as the etiology of Parkinson's disease.

Objective 2:
To examine the effects of chronic stress on basal concentrations of glutamate in the hippocampus.

Rationale: Evidence is accumulating that stress is associated with the onset of depression, a dysregulation of the hypothalamic-pituitary-adrenal axis, and possible neurodegeneration. With regard to the latter, McEwen and colleagues have described a model of stress-induced morphological reorganization in the hippocampus that appears to be mediated by excitatory amino acids and adrenal steroids (McEwen, 1997). Unpublished observations indicate that chronic stress increases mRNA expression of the glial glutamate transporter (GLT-1) in the CA3 region of the hippocampus (Reagan and McEwen) and further support the role of excitatory amino acids in mediating the neurodegeneration observed in this area following chronic stress. Consistent with these findings of stress-induced increases in excitatory amino acid transmission in the hippocampus, we have shown that acute restraint stress increases hippocampal glutamate release measured in vivo; an effect that is reversed by adrenalectomy (Lowy et al., 1993).
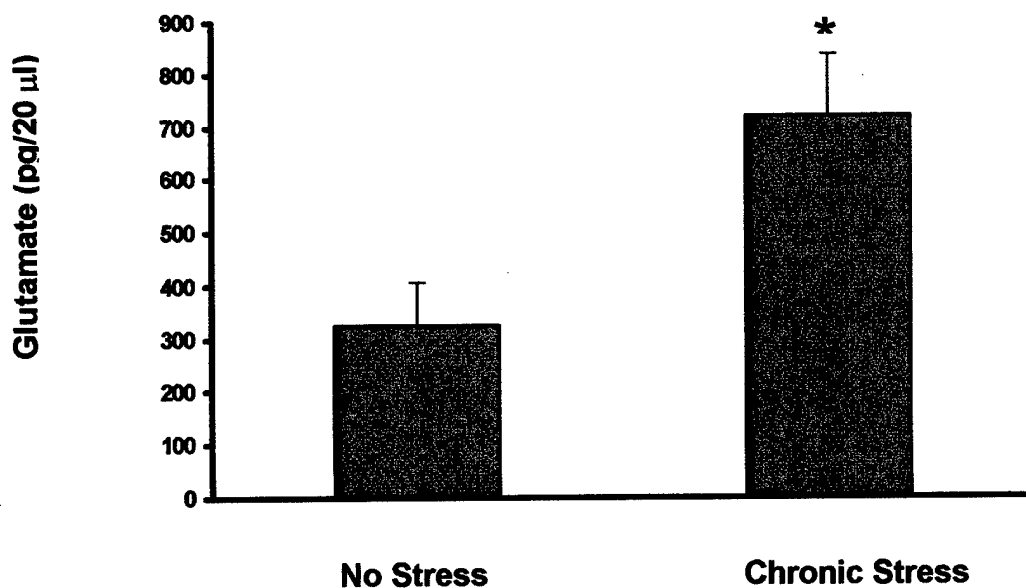
Results:

Fig. 1

Figure 1 shows that chronic restraint stress for 21 days using a commercially available plastic rat restrainer increases the basal concentrations of glutamate in the hippocampus (*$p<0.05$).

Discussion:
These findings illustrate that chronic stress can increase the extracellular concentrations of glutamate and perhaps account for the hippocampal remodeling and apical dendritic atrophy that has been observed by others (McEwen, 1997). Future studies will focus on the mechanisms mediating this increase in extracellular glutamate and will focus on glutamate release processes as well as glutamate uptake systems.

Objective 3:
To examine the effect of methamphetamine on GABA release in the substantia nigra (SN) and the local regulation of GABA by DA as an index of the outflow activity of the basal ganglia.

Rationale: High extracellular concentrations of DA and the excitatory amino acid glutamate (GLU) have been implicated in mediating METH toxicity (Nash and Yamamoto, 1992). Systemic administration of METH increases both DA and GLU release. However, while local perfusions of METH directly into the striatum do produce an increase in DA release they do not produce an increase in GLU and do not produce long term depletions of striatal DA tissue content (Burrows et al., 2000). This suggests that increases in both DA and GLU are necessary to produce neurotoxicity.

Although GLU appears to be significant in mediating METH toxicity, it is still unclear how METH increases GLU. Our hypothesis is that METH will increase extracellular GLU via the striatal outflow pathways, specifically the striatonigral efferents. We predicted that stimulation of the D1 receptors in the SN will increase GABA release in the SN.

Results:
Systemic administration of METH alone produced an increase of extracellular GABA release in the substantia nigra (*$p<0.05$). The local perfusion of D1 antagonist, SCH23390 (SCH), alone into the SN decreased the basal concentrations of GABA and attenuated the METH induced increase of extracellular GABA.

Fig. 2
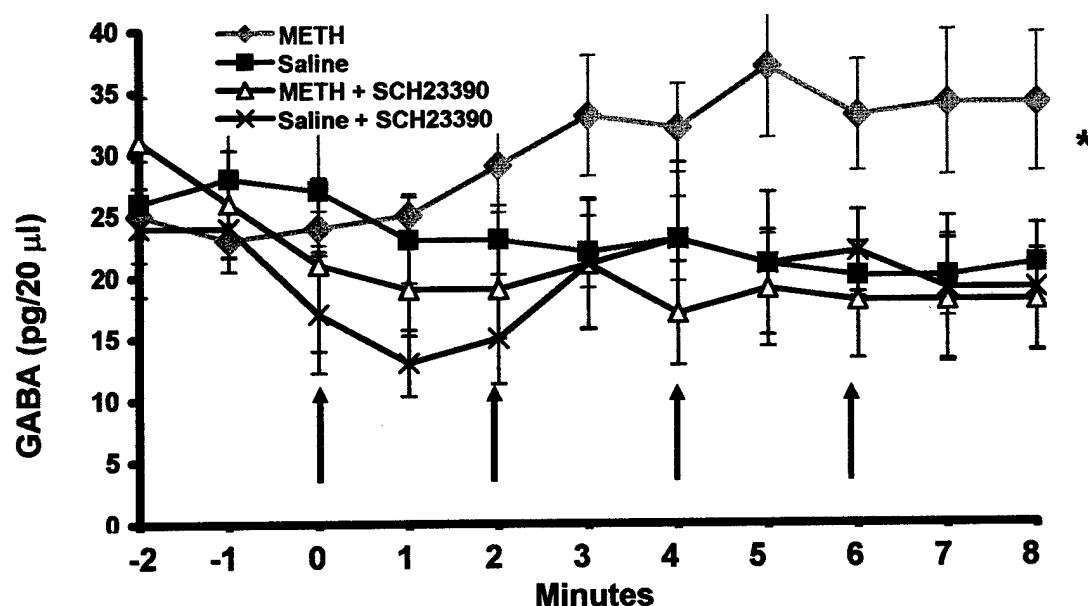
To examine whether the inhibition of GABA release in the substantia nigra pars reticulata can eventually decrease the long-term depletion of dopamine produced by methamphetamine, dopamine content in the striatum was measured 7 days after the dialysis experiment in METH or saline pretreated rats. SCH (METH-SCH group) significantly attenuated (**p<0.05 from METH-Vehicle group) the METH-induced dopamine depletions (*p<0.05; METH pretreated-vehicle infused group) in the striatum.

Fig. 3
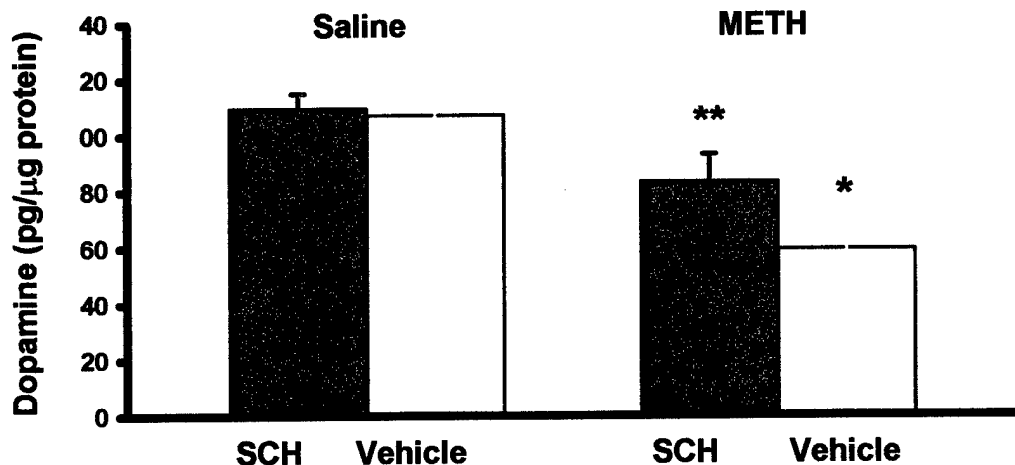
Discussion:
Basal and stimulated GABA release in the SN is modulated by D1 receptors. Furthermore, neurotoxicity to DA terminals in the STR after METH is partially mediated by activation of D1 receptors and GABA release in the SN. It can be postulated from the circuitry model illustrated below that an increase of extracellular GABA in the SN will result in the activation of the thalamocortical projections and a subsequent increase in extracellular GLU release in the striatum.

It remains to be determined if disinhibition of the thalamocortical path via decreased inhibitory input to the thalamus from the SN results in an increase in striatal glutamate release.

Objective 4:

Objective 4a: To characterize the input from the subthalamic nucleus to the substantia nigra.

Results:

Using dual probe microdialysis, we examined the effect of the reverse dialysis of carbachol into the subthalamic nucleus while simultaneously measuring glutamate release in the substantia nigra. As illustrated below, carbachol infusion significantly increased (*p<0.05) glutamate release in the substantia nigra. The increase in glutamate returned to baseline despite the continued perfusion of carbachol

Effect of carbachol (1mM) administration in the subthalamic nucleus on extracellular glutamate concentration in the substantia nigra

Fig. 4

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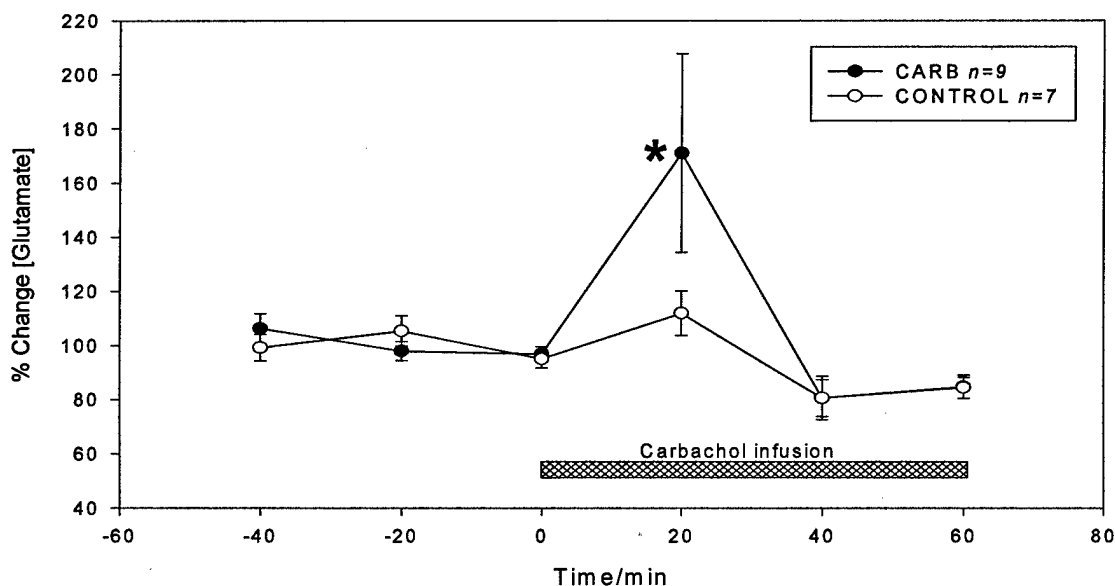
Discussion

These data indicate that stimulation of muscarinic receptors in the subthalamic nucleus results in an increase in subthalamic activity to increase glutamate release in the substantia nigra. we plan to examine the regulation of the subthalamonigral pathway by metabotropic and dopaminergic receptors in the substantia nigra. The rapid return of glutamate towards basal values despite the continued stimulation by carbachol may indicate that there is a negative feedback control of stimulated glutamate release. Objective 4b was to investigate this possibility.

Objective 4b: To examine the modulatory role of dopamine on extracellular glutamate in the substantia nigra.

Rationale: It has been shown that glutamate release in the substantia nigra may be regulated by dopamine and D2 receptors in an inhibitory manner. It is important to understand the modulation of this pathway since it provides and significant excitatory input into the SN. Dysregulation of this pathway may lead to excitotoxicity in the SN and contribute to Parkinson's disease.

Hypothesis: Antagonism of D2 receptors by raclopride in the SN will enhance or prolong the carbachol-induced release of glutamate from subthalamonigral terminals.

Fig. 5

RACLOPRIDE IN THE SN ENHANCES THE CARBACHOL-INDUCED GLUTAMATE RELEASE

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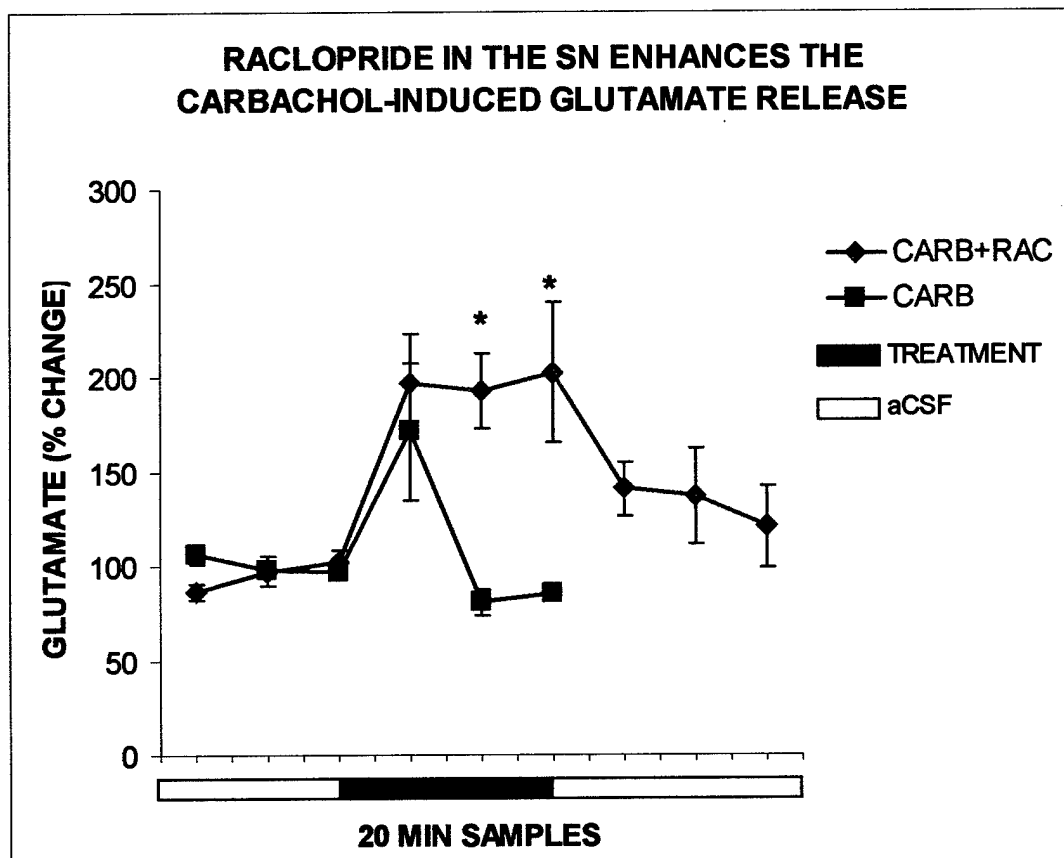
Results:

These data show that perfusion of raclopride in the SN during the perfusion of the subthalamic nucleus with carbachol prolongs the increase in glutamate.

Objective 4c: To examine the extracellular concentrations of glutamate in the substantia nigra after METH in the presence or absence of raclopride perfusion into the SN.

Hypotheses: The perfusion of raclopride into the SN during the systemic administration of METH will increase extracellular glutamate. METH should produce a long-term decrease in dopamine content in the striatum. Furthermore, repeated treatment of the longer acting D2 antagonist, haloperidol for 5 days after the administration of METH will produce not only a decrease in dopamine content in the striatum but also in the substantia nigra.

Resutts:

The systemic administration of METH and raclopride increased the extracellular concentrations of glutamate in the SN (Fig. 6).

FIGURE 6: SYSTEMIC METH + RACLOPRIDE PERFUSION OF THE SN INCREASED THE EXTRACELLULAR CONCENTRATIONS OF GLUTAMATE IN THE SN.
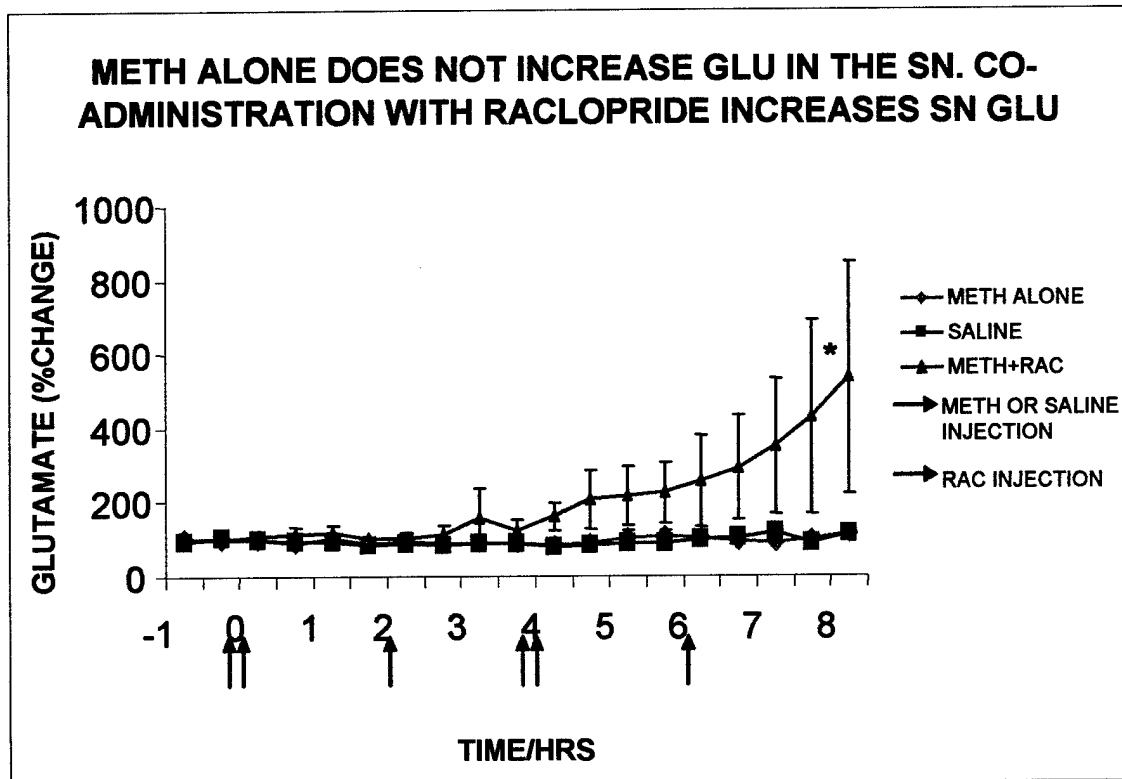
METH alone or the subchronic administration of the D2 antagonist, haloperidol for 5 days after the administration of METH produced a depletion of dopamine content in the striatum (Fig. 7). More importantly, the post-METH administration of haloperidol produce a decrease in dopamine content in the substantia nigra 7 days after the METH treatment (Fig. 8).

Fig. 7

METH DECREASES DA TISSUE CONTENT IN THE STRIATUM
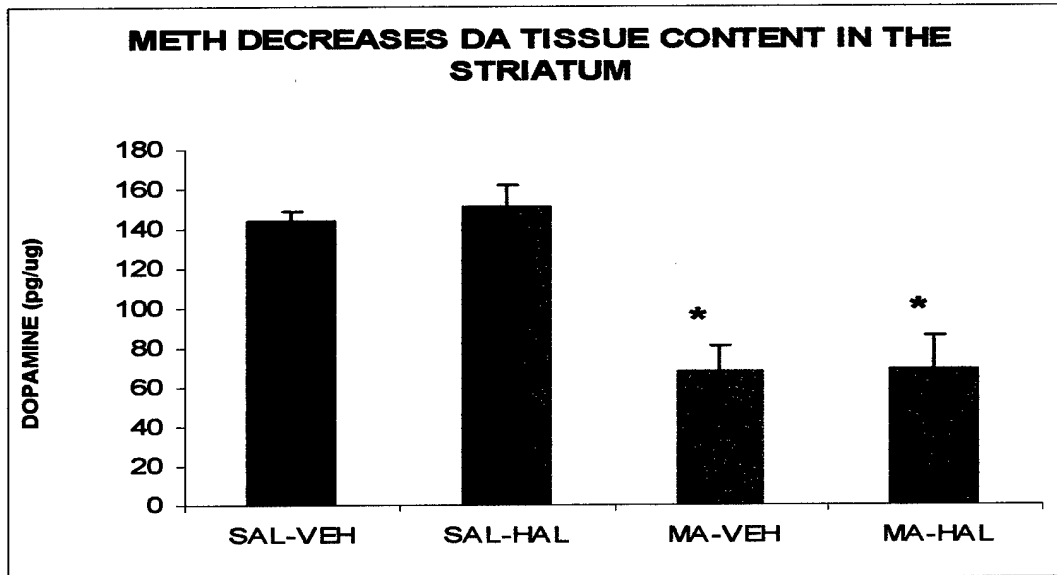
MA=METH; HAL=haloperidol; SAL=Saline; VEH=vehicle

Fig. 8

CHRONIC D2 ANTAGONISM AFTER METH DECREASES DA TISSUE CONTENT IN THE SN
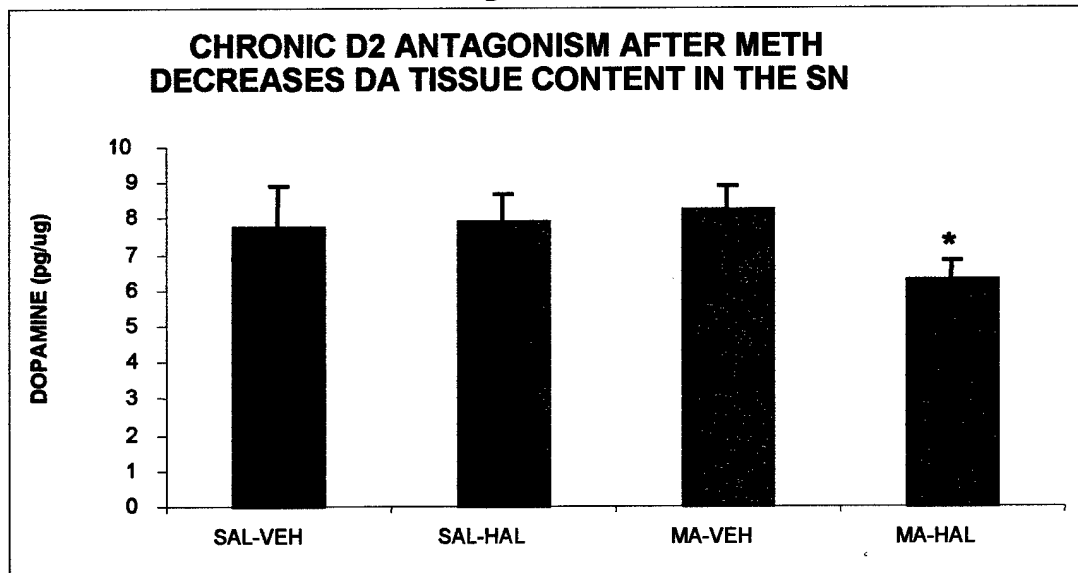
Discussion:

These data indicate that METH by itself does not have neurotoxic effects in the SN as manifested by the lack of a long-term depletion of dopamine content. This

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may be explained by the lack of increase in extracellular glutamate in the SN during the administration of METH. These findings, in combination with the studies in Objective 3 showing that the D1 regulated direct output pathway of the basal ganglia is affected by METH, illustrate that METH primarily affects the D1 direct pathway (Fig. 2) and not the indirect pathway mediated through the subthalmic nucleus (Fig. 6). However, if glutamate release from the subthalamonigral terminals is disinhibited by the antagonism of D2 receptors in the SN (Figs 5 and 6), extracellular glutamate concentrations are increased (Fig 6) which in turn, may produce damage to dopamine cell bodies in the SN (Fig 8). These data have significant implications for METH toxicity and the potential for the development of Parkinson's disease, particular since D2 antagaonists such as haloperidol are used acutely for the emergency treatment of METH-induced psychosis.

Objective 5:
To examine the effect of methamphetamine on oxidative damage in the striatum as measured by protein nitration.

As described in the Progress Report last year, methamphetamine increased the formation of nitrotyrosine in the striatum, as measured by HPLC analysis of acid hydrolyzed protein. To verify this finding with another method, we employed the immunohistochemical detection of an anti-nitrotyrosine antibody 24 hrs after the administration of methamphetamine.

Results:
As can be observed in the figure below, there was increased immunohistochemical staining observed in the striatum of methamphetamine treated rats.

Fig. 9

Saline   Methamphetamine
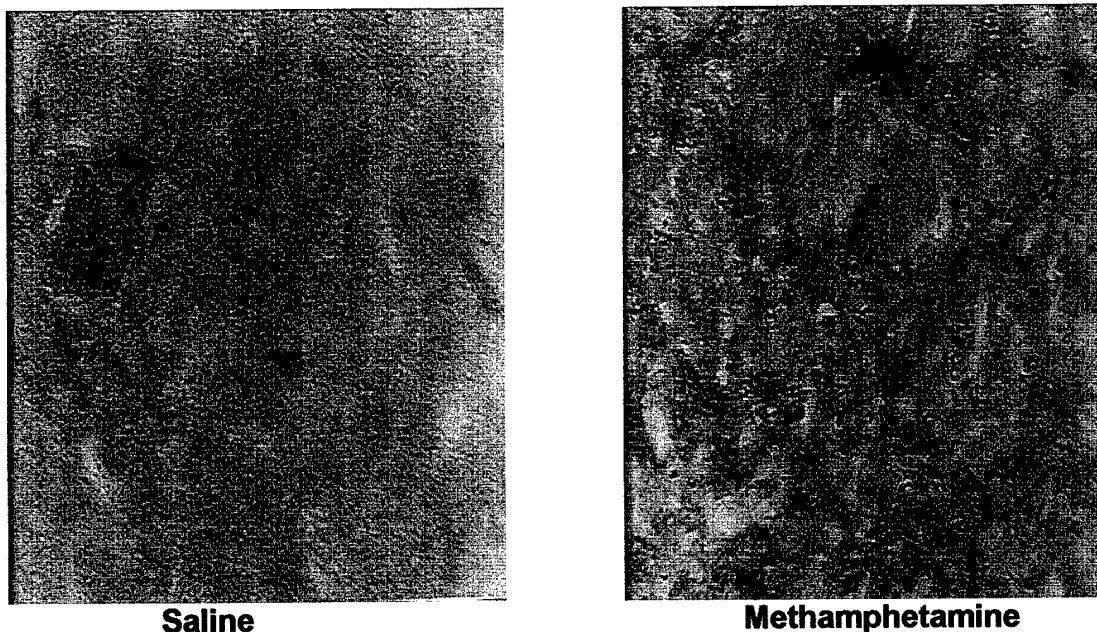
Discussion:

The results indicate that methamphetamine produces evidence of oxidative stress in the striatum as indicated by the nitration of protein in the form of nitrotyrosine. These data are consistent with our previously published studies showing the METH increases the extracellular concentrations of glutamate and dopamine that in turn, could lead to the formation of glutamate-induced nitric oxide and dopamine-derived production of hydrogen peroxide, both of which can combine to form peroxynitrite and the subsequent nitration of protein.

Objective 6:

To examine the effects of METH on the activity and protein content of electron transport chain complexes of the mitochondria.

Results:

Complex II-III but not I-III activities decreased the activity of 1 hr after the last (4th) injection after methamphetamine (4 X 10 mg/kg ip) (Fig. 10). This effect was restricted to the striatum and was not seen in the hippocampus (Fig. 11).

Fig. 10

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Fig. 11

Striatum

Hippocampus
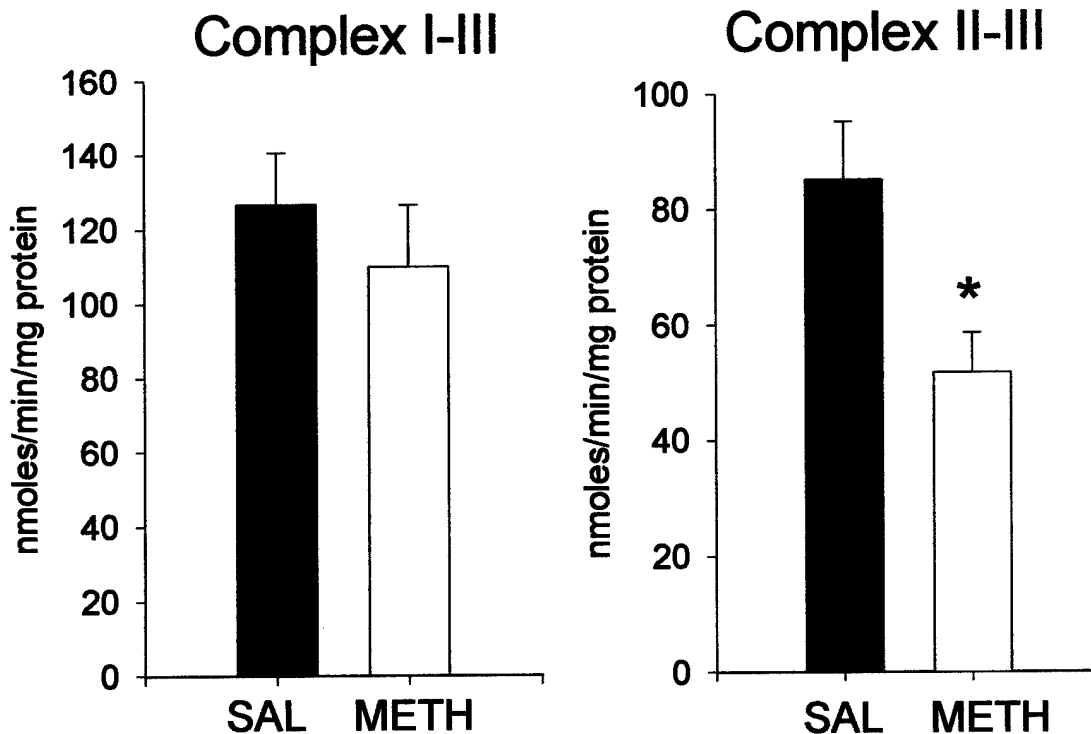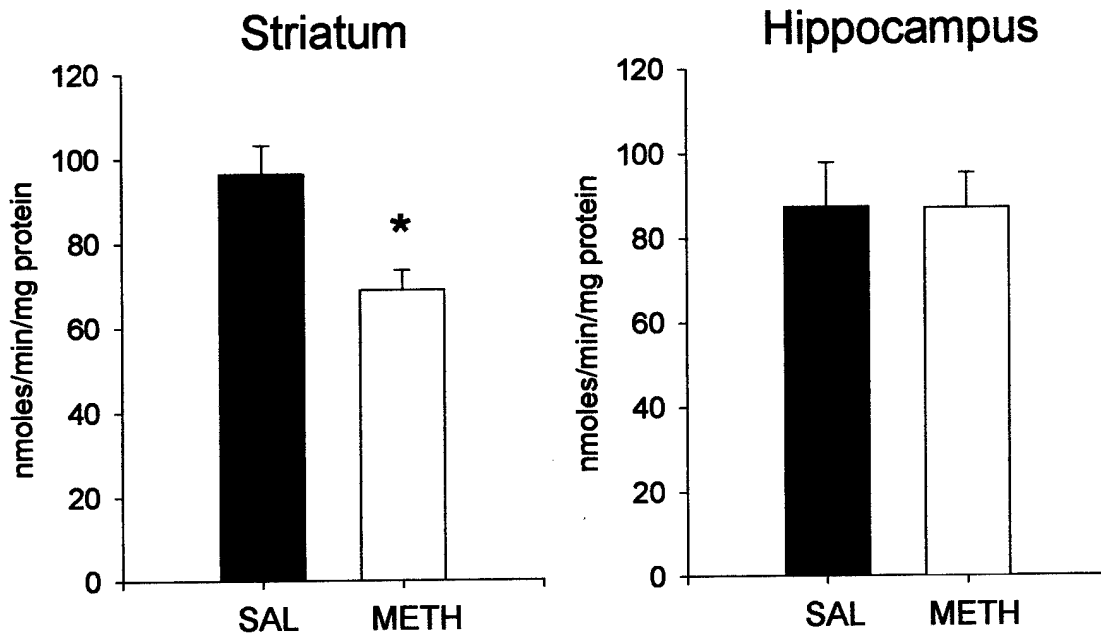
To determine if the decrease in Complex II-III activity was due to the hyperthermic effect of METH, rats were maintained at normothermic temperatures (37°C) during the METH treatment. Complex II-III activity was still decreased despite cooling to maintain normothermia (Fig. 12).

Fig. 12
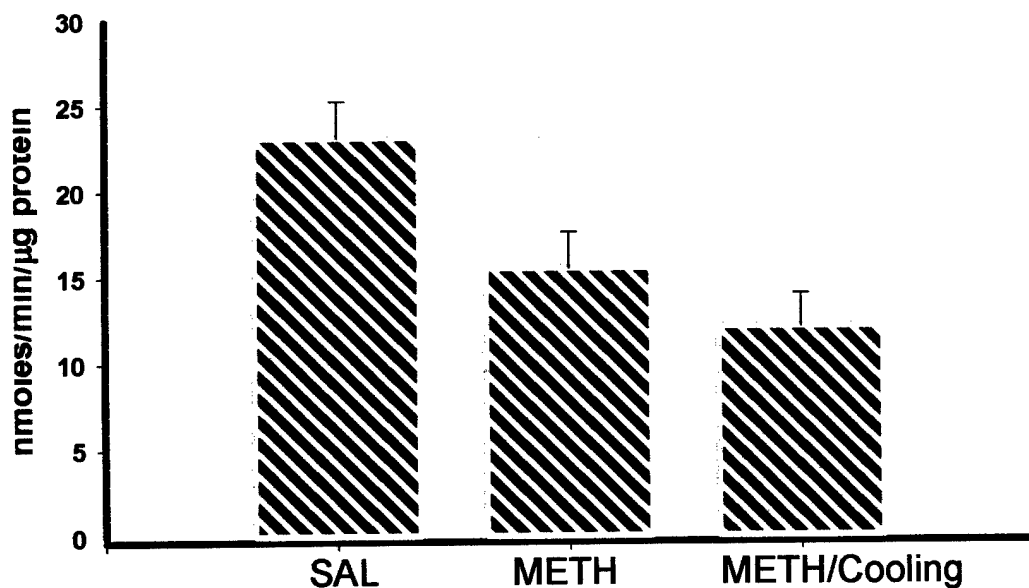
To examine if the decrease in Complex II-III activity was a result of a direct effect of METH on the mitochondria, mitochondria were incubated directly with varied concentrations of METH and Complex II-III activity was measured. Complex II-III activity was only decreased at high millimolar concentrations.

Fig. 13
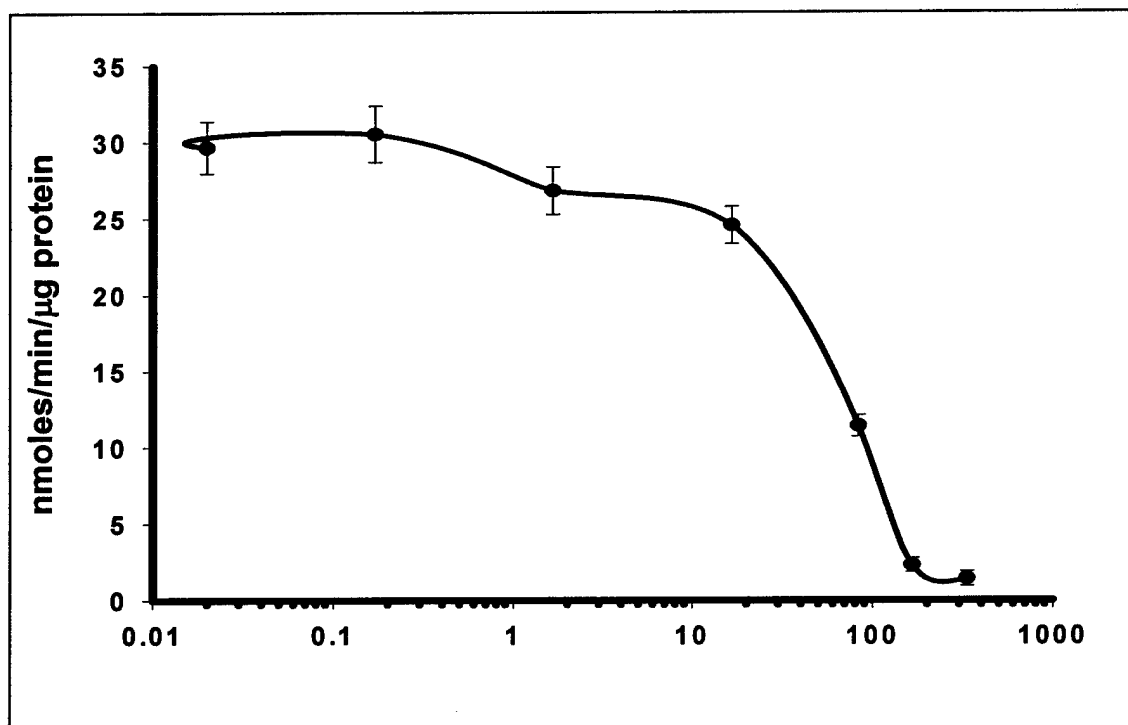
When Complex II activity (succinate dehydrogenase) was selectively measured, there also was a decrease 1 hr after METH. Moreover, the decrease was blocked by the NMDA receptor antagonist MK801 (Fig. 14).

Fig. 14
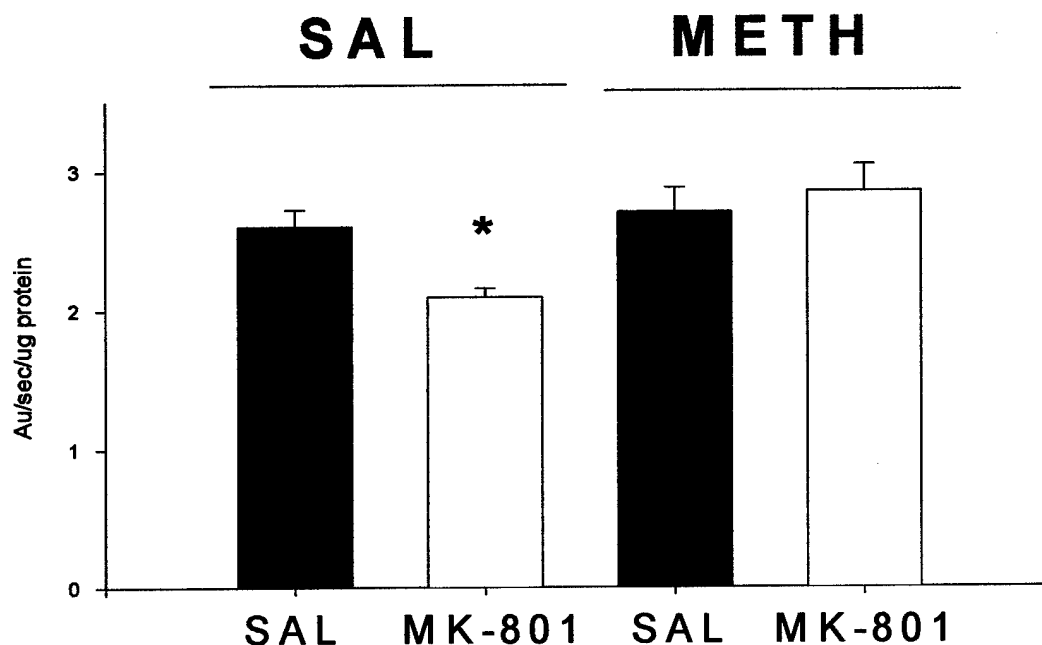
To determine if the decrease in Complex II activity was due to a loss of the enzyme protein, the immunoreactivities of catalytic 70 kD subunit and the 30 kD subunit were measured. METH produce a decrease in the immunoreactivity of both the 70 kD and 30 kD subunits of Complex II (Fig. 15).

Fig. 15
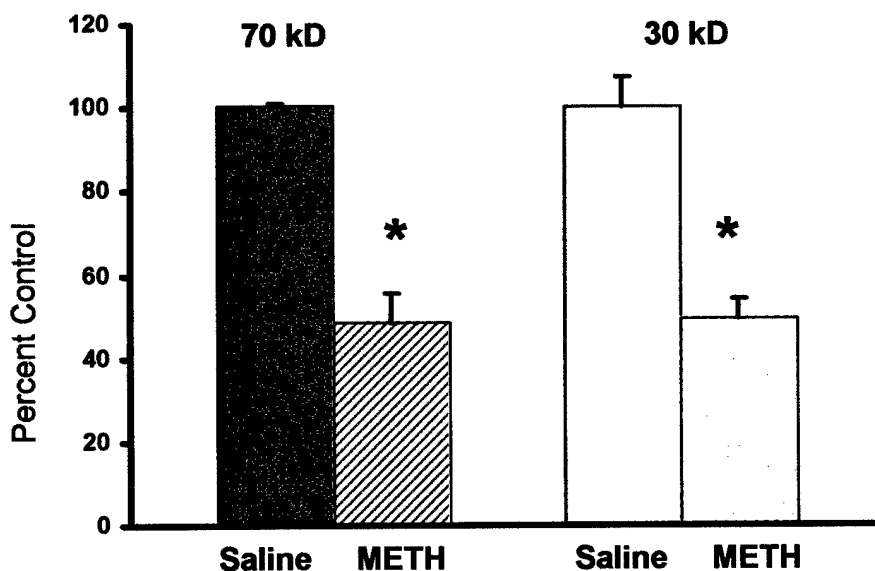
Discussion: These data indicate that METH produces a selective and rapid inactivation of Complex II of the mitochondria. This effect is not due to a direct effect of METH since only at mM concentrations of METH was there a decrease in enzyme activity when METH was directly incubated with the mitochondrial preparation, at least 100 times higher than what is achieved in the brain following a neurotoxic regimen with repeated systemic administrations. Moreover, the decrease was not due to the hyperthermic effects of METH since cooling the rats had no effect on the decrease in activity. The decrease in Complex II enzyme activity is most likely due to a degradation of the enzyme as evidenced by the decrease in protein subunit immunoreactivity.and is mediated by glutamate and the NMDA receptor.

Objective 7:
Objective: To examine the cause of the chronic stress-induced exacerbation of METH-induced hyperthermia described in Objective 1.

Results:
Rats exposed to 10 days, but not 2 days, of unpredictable stress exhibited higher rectal temperatures following the $5HT_{2A/2C}$ agonist, DOI than non-stressed rats. The DOI-induced hyperthermia was attenuated by the $5HT_2$ antagonist, LY-53,587. The augmentation of DOI-induced hyperthermia in stressed rats persisted when examined 8, 30 and 60 days following the stress procedure.

See attached manuscript: Matuszewich, L, and Yamamoto, B.K Long-lasting effects of chronic stress on DOI-induced hyperthermia. Psychopharmacology 169: 169-175, 2003.

Discussion:
The enhancement of 5-HT receptor function by chronic stress persists even after the environmental stressor is removed. These data indicate that chronic stress may be exacerbating the METH-induced hyperthermia through and 5HT2-dependent mechanism. This lasting increase in 5-HT receptor function may have implications for clinical disorders associated with stress, such as depression or post-traumatic stress disorder.

Objective 8:

To examine the excitotoxic effects of methamphetamine as measured by calpain-mediated spectrin proteolysis. Spectrin breakdown products were measured by western blot analysis. Representative 145 kD immunoreactive bands of spectrin breakdown products are noted below the bars (Fig. 16).

METH increased the immunoreactivity of spectrin breakdown products and the NMDA antagonist, MK 801 administered 5 hrs after METH blocked spectrin proteolysis.

Fig. 16
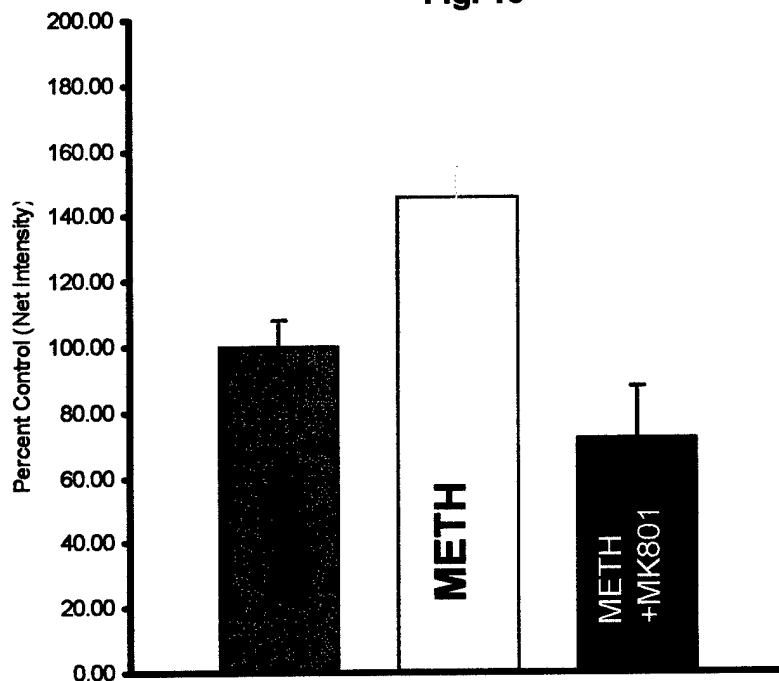
KEY RESEARCH ACCOMPLISHMENTS

♦ Chronic unpredictable stress enhances the neurotoxicity of methamphetamine to dopamine terminals

♦ Chronic restraint stress elevates basal extracellular glutamate concentrations in the hippocampus and may mediate the damage to this area.

♦ Chronic stress enhances the hyperthermic responses to the $5HT_{2A/C}$ agonist, DOI and thus may indicate that chronic stress can exacerbate hyperthermia via a sensitization of 5HT2 receptors.

♦ Methamphetamine increases GABA release in the substantia nigra via the D1 receptor and indicates the direct output pathway of the basal ganglia is activated. The increase in GABA release in the substantia nigra plays a critical role in the long-term loss of dopaminergic innervation to the striatum following methamphetamine.

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- ◆ Methamphetamine produces protein oxidation and excitotoxicity in the striatum as evidenced by ntrotyrosine immunoreactivity and spectrin proteolysis, respectively.
- ◆ Methamphetamine produces a bioenergetic compromise as evidenced by a decrease in complex II activity and protein.
- ◆ An experimental design and methodology was established to study *the in vivo* regulation of the glutamatergic projection from the subthalamic nucleus to innervate the substantia nigra. METH in combination with the antagonism of D2 receptors in the SN elevates the extracellular concentrations of glutamate and produces a long-term depletion of dopamine in the SN.

REPORTABLE OUTCOMES

Published papers

Matuszewich, L., Filon, M.E., Finn, D.A., and Yamamoto, B.K. Altered forebrain neurotransmitter responses to immobilization stress following 5-HT depletions with MDMA. Neuroscience 110: 41-48, 2002

Brown, J. and Yamamoto, B. Psychostimulants and mitochondrial function. Pharmacology and Therapeutics 99: 45-53, 2003.

Matuszewich, L, and Yamamoto, B.K Long-lasting effects of chronic stress on DOI-induced hyperthermia. Psychopharmacology 169: 169-175, 2003.

Matuszewich, L. and Yamamoto, B.K. Chronic Stress Augments the Acute and Long-term Effects of Methamphetamine. Neuroscience 124: 637-646, 2004.

Submitted Paper

Brown, J.M. and Yamamoto, B.K. A rapid and selective decrease in mitochondrial complex II activity and protein subunits by methamphetamine.

Abstracts

Matuszewich, L. and Yamamoto, BK, Chronic unpredictable stress produces persistent DOI-induced hyperthermia in rats. Society for Neuroscience, 2001

Gallaugher, L, Stamm, M and Yamamoto, BK Dopamine-GABA interactions in the substantia nigra: Effects of methamphetamine. Society for Neuroscience, 2001

Matuszewich, L and Yamamoto, BK "Effects of Chronic Stress on Methamphetamine-Induced Dopamine Depletions in the Striatum" Annual International Society for Psychoneuroendocrinology Conference in New York City, September 7-9, 2003.

Mark KA, Soghomonian JJ, and Yamamoto, BK Methamphetamine and GABAergic transmission in the basal ganglia. Society for Neuroscience, 2003

Hatzipetros T, Raudensky J, and Yamamoto B Dopaminergic Regulation of Glutamate Transmission in Rat Subthalamonigral Path. Society for Neuroscience, 2003

Brown, JM and Yamamoto, BK High-dose methamphetamine administration inhibits complex II of the mitochondrial electron transport chain. Society for Neuroscience, 2003

Brown, JM and Yamamoto BK High-dose methamphetamine administration inhibits striatal mitochondrial electron transport complexes. FASEB, 2003

CONCLUSIONS

The experiments conducted in accordance with the
Statement of Work revealed several interesting outcomes. The results illustrated
in this progress report support the conclusion that chronic environmental stress
can potentiate the neurotoxic effects of methamphetamine on dopamine
terminals. The mechanisms underlying the enhanced vulnerability produced by
stress on methamphetamine-induced damage to dopamine neurons may include
excitotoxicity mediated by an enhanced release of glutamate in the striatum or
substantia nigra. The additive or synergistic effects of stress and
methamphetamine-induced insults may enhance the vulnerability to the
development of Parkinson's disease.

In addition, other findings described in this progress report have begun to
characterize how the release of dopamine within the substantia nigra can alter
the outflow of the basal ganglia through the activation of D1 receptors that in
turn, could lead to an increase in corticostriatal glutamate release via a
polysynaptic process to culminate in excitotoxic damage (spectrin proteolysis) to
dopamine terminals in the striatum and through D2 receptor inhibition within the
substantia nigra to disinhibit glutamate release and damage dopamine soma.

We have established a methodology to stimulate glutamate efferents from the
subthalamic nucleus that innervate the substantia nigra while simultaneously
measuring glutamate release. As a result, we are now positioned to determine if
(1) chronic stress exacerbates this increase in glutamate release and enhances
the vulnerability of dopamine soma in the substantia nigra to excitotoxicity and
oxidative damage and (2) the regulatory mechanisms of the glutamatergic
efferents of the subthalamic nucleus to the substantia nigra. Overall, these
studies continue to address the hypothesis that the synergistic interaction
between chronic stress and methamphetamine damages dopamine neurons at
both the terminal (striatum) and cell body (substantia nigra).

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Nash J.F. and Yamamoto, B.K. Methamphetamine neurotoxicity and striatal
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LIST OF APPENDICES

Matuszewich, L., Filon, M.E., Finn, D.A., and Yamamoto, B.K. Altered forebrain neurotransmitter responses to immobilization stress following 3,4-Methylenedioxymethamphetamine. Neuroscience 110: 41-48, 2002.

Matuszewich, L, and Yamamoto, B.K Long-lasting effects of chronic stress on DOI-induced hyperthermia in male rats. Psychopharmacology 169: 169-175, 2003.

Matuszewich, L. and Yamamoto, B.K. Chronic stress augments the long-term and acute effects of methamphetamine. Neuroscience 124: 637-46, 2004.

Brown, J.M. and Yamamoto, B.K. Effects of amphetamines on mitochondrial function: role of free radicals and oxidative stress. Pharmacology and Therapeutics 99: 45-53, 2003.

Brown, J.M. and Yamamoto, B.K. A Rapid and Selective Decrease in Mitochondrial Complex II Activity by Methamphetamine, submitted for publication.

APPENDIX 1

ALTERED FOREBRAIN NEUROTRANSMITTER RESPONSES TO IMMOBILIZATION STRESS FOLLOWING 3,4-METHYLENEDIOXYMETHAMPHETAMINE

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Abstract—(±)3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is an increasingly popular drug of abuse that acts as a neurotoxin to forebrain serotonin neurons. The neurochemical effects of the serotonin depletion following high doses of MDMA were investigated in response to acute immobilization stress. Male rats were treated with a neurotoxic dosing regimen of MDMA (10 mg/kg, i.p. every 2 h for four injections) or equivalent doses of saline. Seven days after treatment, *in vivo* microdialysis was used to assess extracellular dopamine and serotonin in the dorsal hippocampus and prefrontal cortex during 1 h of immobilization stress. In saline treated control rats, serotonin in the hippocampus and serotonin and dopamine in the prefrontal cortex were increased during immobilization stress. Rats pretreated with MDMA, however, showed blunted neurotransmitter responses in the hippocampus and the prefrontal cortex. In the drug pretreated rats, basal serotonin levels in the hippocampus, but not the prefrontal cortex, were lower compared to saline pretreated controls. Stress-induced increases in plasma corticosterone and body temperature were not affected by the pretreatment condition.

From these studies we suggest that depletion of serotonin stores in terminal regions with the neurotoxin MDMA compromises the ability of the serotonergic neurons to activate central systems that respond to stressful stimuli. This altered responsiveness may have implications for long-term functional consequences of MDMA abuse as well as the interactions between the serotonergic system and stress. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: MDMA, immobilization stress, prefrontal cortex, hippocampus, serotonin, dopamine.

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is a commonly abused 'club drug' with long-term neurochemical effects on the CNS. Administration of high doses of MDMA results in depletions of serotonin (5-hydroxytryptamine or 5-HT) in the frontal cortex, hippocampus, and striatum of rodents and non-human primates (Callahan et al., 2001; Gibb et al., 1990; Schmidt, 1987; Stone et al., 1986). Neuronal damage to the serotonergic system is evidenced by decreased tryptophan hydroxylase activity, decreased number of 5-HT reuptake sites and immunocytochemical damage to 5-HT axons following high doses of MDMA (Battaglia et al., 1987; Commins et al., 1987; O'Hearn et al., 1988; Schmidt and Taylor, 1987; Stone et al., 1987). The degeneration of 5-HT axons has been reported to be limited to fine-diameter axons from the dorsal raphe nucleus that project to forebrain regions, such as the hippocampus and frontal cortex (Mamounas et al., 1991; O'Hearn et al., 1988; Wilson et al., 1989).

Although the neurochemical alterations following neurotoxic treatment with MDMA have been well characterized, the functional consequences of MDMA at neurotoxic doses are relatively unknown. Several studies have reported that 7 days after treatment with a neurotoxic regimen of MDMA, extracellular 5-HT responses to an acute injection of MDMA or D-fenfluramine are suppressed, as are behavioral and hyperthermic responses (Series et al., 1994; Shankaran and Gudelsky, 1999). Prior exposure to a neurotoxic regimen of MDMA also decreased the sensitivity of rhesus monkeys to the subsequent effects of an acute injection of dexfenfluramine on a behavioral task, or of rats to MDMA in a

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drug discrimination task (Frederick et al., 1995; Schechter, 1991). However, in a behavioral paradigm without the use of a drug challenge, MDMA pretreatment in rats had no effect on a place navigational learning-set task, or maze performance (Ricaurte et al., 1993; Robinson et al., 1993; Seiden et al., 1993; Slikker et al., 1989). Thus, the effects of MDMA on the functioning of the CNS may only be apparent during an activated or stimulated state, such as following a drug injection or an environmental stressor.

The integrity of the central serotonergic system appears to be important for some types of neurochemical responses to the presentation of an acute stressor. In rats, lesions of hypothalamic 5-HT neurons with 5,7-dihydroxytryptamine (5,7-DHT) attenuate the increase in plasma corticosterone concentrations following photic stimuli (Feldman, 1985; Feldman et al., 1984). In contrast, dopamine metabolism in prefrontal cortex and nucleus accumbens following 30 min of restraint stress was enhanced in rats that received injections of 5,7-DHT into the dorsal raphe (Morrow and Roth, 1996). Therefore, a sufficient serotonergic tone may be important in maintaining the appropriate neurochemical responses to an acute stressor.

Exposure to an environmental stressor increases 5-HT and dopamine levels in several brain regions, including areas innervated by 5-HT and depleted by MDMA (for review see Chaouloff, 1993). The application of acute stressors has been correlated with increased 5-HT release in the prefrontal cortex, hippocampus, striatum, hypothalamus, amygdala and periaqueductal gray of the rat as measured by *in vivo* microdialysis or voltammetry (Adell et al., 1997; Amat et al., 1998; Boutelle et al., 1990; Joseph and Kennett, 1983, 1986; Kawahara et al., 1993; Kirby et al., 1997; Shimizu et al., 1992; Takahashi et al., 1998; Yoshioka et al., 1995). In a similar manner, extracellular dopamine and dopamine turnover increase in the prefrontal cortex, striatum and nucleus accumbens in response to tail shock/pressure, restraint, foot shock or handling stress (Abercrombie et al., 1989; Cenci et al., 1992; Finlay et al., 1995; Finlay and Zigmond, 1997; Gresch et al., 1994; Imperato et al., 1991; Kawahara et al., 1999; Keefe et al., 1993; Nakahara and Nakamura, 1999; Sorg and Kalivas, 1993; Taber and Fibiger, 1997; Thierry et al., 1976). The hippocampus and prefrontal cortex may play important roles in the interaction between neurotransmitter and neuroendocrine responses to stress due to the large number of corticosterone receptor sites in each, their projections to hypothalamic structures, and their dense monoamine innervation (Chaouloff, 1993; Diorio et al., 1993; Herman and Cullinan, 1997; McEwen et al., 1986).

It remains to be determined whether 5-HT terminal damage produced by exposure to high doses of MDMA alters the stress-induced release of monoamines in brain regions targeted by MDMA. The objective of the present study was to measure extracellular concentrations of 5-HT in the hippocampus, as well as 5-HT and dopamine in the prefrontal cortex, during immobilization stress of rats that were administered MDMA 7 days earlier.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (175–250 g) were purchased from Zivic Miller Labs (Allison Park, PA, USA). Rats were housed individually, with food and water available *ad libitum*, on a 12-h light–dark cycle in a temperature controlled room. All procedures were in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the local institutional animal care committee.

Drugs

All rats were given i.p. injections with 10 mg/kg MDMA hydrochloride salt (National Institutes of Drug Abuse, Bethesda MD, USA) or an equivalent volume of saline (0.9% NaCl), every 2 h for a total of four injections. Drug injections were given in a volume of 1 ml/kg. The above injection procedure has been shown previously to decrease 5-HT neurotransmitter content in the striatum (Shankaran and Gudelsky, 1999).

Surgical procedures

Three days after the i.p. injections, all rats were anesthetized with a combination of xylazine (6 mg/kg) and ketamine (70 mg/kg) and placed into a Kopf stereotaxic frame. The skull was exposed and two 21-gauge stainless steel guide cannulae (Small Parts, Miami Lakes, FL, USA) were positioned above the cortex (prefrontal cortex: 3.2 mm anterior and 0.5 mm medial to bregma) and dorsal hippocampus (3.2 mm posterior and 2.0 mm medial to bregma). The cannulae and a metal female connector were secured to the skull with three stainless steel screws and cranioplastic cement. Obturators fashioned from 31-gauge stainless steel wire, ending flush with the guide cannulae, were inserted into the cannulae after surgery.

Experiment 1: Measurement of monoamines with in vivo microdialysis during restraint stress

Three days after surgery, the obturators were removed from the guide cannulae and replaced with microdialysis probes. The probes were constructed as previously described (Lowy et al., 1993) from a 27-gauge thin wall stainless steel tube, fitted with a dialysis membrane (13 000 Da cut off, 210 μm outer diameter; Spectrum Laboratories, Rancho Domingues, CA, USA) at one end, and a 3 cm piece of polyethylene 20 tubing (Fisher Scientific, Pittsburg, PA, USA) at the other end, to serve as the inlet for the perfusion medium. The dialysis membrane was 4 mm or 2 mm×210 μm diameter for the prefrontal cortex or dorsal hippocampus, respectively. A 4 cm length of capillary tubing (125 μm outer diameter, 50 μm inner diameter; Polymicro Technologies, Phoenix, AZ, USA) served as the outlet from the dialysis membrane. The vertical placement of the microdialysis probe was determined during construction of the probe by gluing a ring of polyethylene 20 tubing, which acts as a mechanical 'stop', at a measured distance along the length of the probe. The positioning permitted the exposed portion of the dialysis membrane to extend beyond the guide cannulae and into the prefrontal cortex (ventral from dura −1.0 to −5.0) or dorsal hippocampus (ventral from dura −2.0 to −4.0). The rats were placed in microdialysis cages and attached to a swivel (Instech Laboratories, Plymouth Meeting, PA, USA), with food and water available *ad libitum*.

Eighteen hours after probe insertion, Dulbecco's phosphate-buffered saline medium (138 mM NaCl, 2.1 mM KCl, 0.5 mM

MgCl$_2$, 1.5 mM KH$_2$PO$_4$, 8.1 mM NaHPO$_4$, 1.2 mM CaCl$_2$, and 5 mM D-glucose, pH 7.4) was pumped through the microdialysis probes with a Harvard Model 22 syringe infusion pump (Hollison, MA, USA). The Dulbecco's medium was perfused at a rate of 1.5 µl/min. A 3-h perfusion period was allowed prior to sample collection. Twenty-minute samples were then collected during the following conditions: three baseline samples, three samples during immobilization stress, and three samples following the immobilization stress. The immobilization stress procedure was initiated between 11:30 h and 13:00 h. For the immobilization stress, rats were placed with their ventral surface on a Plexiglas board and secured with a 2-inch Velcro strap across their midregion and a 1-inch Velcro strap behind their head. Tape was used to secure their paws. In five saline and five MDMA pretreated rats, rectal temperatures were monitored every 10 min during the 1-h immobilization stress with a Thermalert TH-8 monitor (Physitemp Instruments, Clinton, NJ, USA). After the 1-h immobilization, the tape and Velcro strips were removed and the rats returned to their microdialysis cages for the remaining three samples.

Experiment 2: Plasma corticosterone concentrations prior to and during restraint stress

Separate groups of rats given injections of either MDMA or saline were killed and trunk blood assayed for corticosterone concentrations. These rats did not have microdialysis cannulae implanted. One week after injections, saline ($n = 12$) or MDMA treated ($n = 12$) rats were killed by rapid decapitation either without immobilization stress ($n = 12$) or 10 min into the immobilization stress procedure ($n = 12$). Trunk blood was collected into a 4-ml vial with 0.1 ml heparin sodium sulfate (1000 U/ml, Pharmacia&Upjohn, Kalamazoo, MI, USA) and centrifuged for 15 min (4000×g).

Histology

After the microdialysis study or the blood collection, brains were quickly removed from the skull and frozen on dry ice. For the microdialysis experiment, probe placements were verified from frozen coronal sections and only rats with probes located in the prefrontal cortex or dorsal hippocampus were used for statistical analysis.

Neurochemical analysis of monoamines (high-performance liquid chromatography)

Microdialysis samples were assayed for dopamine and/or 5-HT by high-performance liquid chromatography with electrochemical detection (HPLC-EC). Samples from the prefrontal cortex were assayed for dopamine and 5-HT, and samples from the hippocampus were assayed for 5-HT only. Samples (20 µl) were loaded via a Rheodyne injector (Cotati, CA, USA) onto a 3-µm C18 column (100×2.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase (pH 4.2) consisted of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM EDTA, 0.215 mM octyl sodium sulfate and 3% methanol. Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), with a 6 mm glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were collected using a Hewlett Packard Integrator.

Analysis of 5-HT and dopamine tissue concentrations of the

frontal cortex and 5-HT tissue concentrations of the dorsal hippocampus were performed on an HPLC-EC, as described above for the monoamines. The tissues were sonicated in 0.1 M perchloric acid, centrifuged at 13 000×g for 6 min. The supernatant was assayed for 5-HT and dopamine. The pellet was resuspended in 1 N NaOH and protein content determined by a Bradford Protein Assay.

Radioimmunoassay of corticosterone

Corticosterone was measured in plasma (5 µl) samples that were diluted with 100 µl sterile water and stored at 4°C until assayed. The radioimmunoassay was adapted from a previously reported procedure (Keith et al., 1978) and employed [^{125}I]corticosterone from ICN Pharmaceuticals (Costa Mesa, CA, USA) and antisera from Ventrex (Portland, ME, USA). Counts per minute were normalized and fit to a least-squares regression equation produced by log–logit transformation of the standards. Mass of samples was calculated by interpolation of the standards. The detectable range of the assay was from 0.1 to 400 µg corticosterone per 100 ml plasma. Intra- and inter-assay coefficients of variation were less than 10%. The specificity of the assay is very high, with only 4% cross-reactivity to deoxycorticosterone, 1% cross-reactivity to 5β-pregnanedione, and less than 0.6% cross-reactivity to other endogenous steroids.

Statistical analysis

Thirty rats were used for the microdialysis experiment. Of the 15 rats treated with MDMA and the 15 rats treated with saline prior to surgery, six rats were excluded from data analysis due to misplaced cannulae or microdialysis difficulties. Further data were excluded from analysis if the average basal monoamine concentrations were below a 3:1 signal-to-noise limit of detection. Twenty-four rats were used to determine the concentration of corticosterone in plasma.

Two-way, repeated-measures analyses of variance (ANOVAs) were computed to compare rats pretreated with MDMA to those pretreated with saline, across all samples (baseline samples, stress samples and post-stress samples) for each neurotransmitter. Individual one-way repeated-measures ANOVAs were also computed separately when appropriate for the saline and MDMA pretreated groups to assess possible differences due to immobilization stress. Post-hoc Newman–Keuls pairwise tests were used to analyze any significant treatments. Independent t-tests were used to compare the pretreatment groups (MDMA vs. saline) on the following measures: 5-HT and dopamine tissue content in the frontal cortex or the dorsal hippocampus; the average of the baseline samples of each neurotransmitter; and peak rectal temperatures. A two-way ANOVA was used to compare the corticosterone plasma concentrations prior to and during immobilization stress, in saline and MDMA pretreated rats. Statistical significance was fixed at $P < 0.05$ for all tests.

RESULTS

Neurochemical content of monoamines

Serotonin tissue content of rats treated with MDMA

Table 1. Serotonin and dopamine tissue levels (µg/g protein) in the dorsal hippocampus and frontal cortex 7 days following treatment with MDMA (10 mg/kg i.p.×4 injections, every 2 h) or an equivalent volume of saline

Brain region and neurotransmitter measured	Saline treated	MDMA treated
Hippocampus 5-HT	2.38 ± 0.15	1.08 ± 0.13*
Frontal cortex 5-HT	2.44 ± 0.30	1.27 ± 0.22*
Frontal cortex dopamine	0.82 ± 0.18	0.72 ± 0.07

All data are presented as the mean ± S.E.M. *$P < 0.05$ vs. saline injected control by an independent Student's t-test.
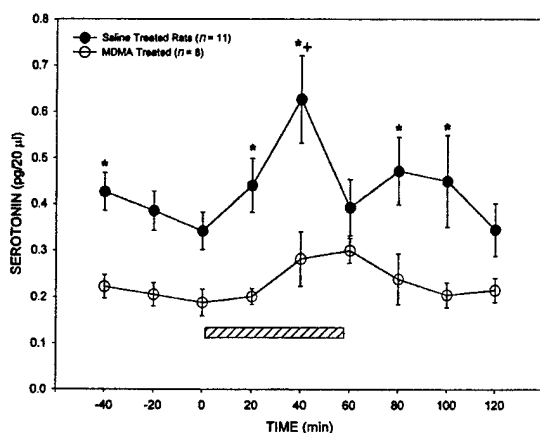
Fig. 1. Effects of 1-h immobilization stress on extracellular levels of 5-HT in the dorsal hippocampus of rats pretreated 7 days earlier with MDMA (10 mg/kg i.p. for four injections, every 2 h) or an equivalent volume of saline. Baseline microdialysis samples were collected for 1 h, after which rats were immobilized (hatched bar) for 1 h followed by a 1-h post-stress interval. Basal concentrations of 5-HT were higher in saline compared to MDMA pretreated rats ($P < 0.05$). Concentrations of 5-HT were elevated in saline pretreated rats during the second period of immobilization stress (time = 40 min) compared to the final baseline (time = 0 min) ($*P < 0.05$ versus respective MDMA pretreated group; $+P < 0.05$ versus time = 0 of saline treated group).

was significantly decreased by 55% in the dorsal hippocampus ($t(28) = 6.28$, $P < 0.01$) and 48% in the frontal cortex ($t(28) = 3.09$, $P < 0.01$) compared to saline treated rats (Table 1). There was no effect of MDMA pretreatment on the concentration of dopamine in the frontal cortex ($t(26) = 0.674$).

Extracellular serotonin response to stress

In the dorsal hippocampus, there was a significant difference between the treatment groups in 5-HT concentrations throughout the experiment as indicated by the main effect of treatment ($F(1,109) = 14.63$, $P < 0.001$; Fig. 1) and through post-hoc comparisons at −40, 20, 40 80 and 100 min ($P < 0.05$). This difference was highlighted by a significant increase in extracellular 5-HT after 40 min of the immobilization stress compared to baseline (time = 0 min) in saline treated rats ($F(8,109) = 2.46$, $P < 0.05$), but not of MDMA treated rats.

In the prefrontal cortex, there was a significant interaction between drug pretreatment (saline or MDMA) across time for extracellular 5-HT concentrations ($F(8,147) = 2.52$, $P < 0.05$, Fig. 2). Post-hoc comparisons indicate that there was a significant increase in 5-HT in the saline, but not the MDMA pretreated group, during the first 20 min of immobilization stress compared to baseline.

Extracellular dopamine response to stress

There was an overall increase in extracellular dopamine concentrations in the prefrontal cortex ($F(8,105) = 2.33$, $P < 0.05$). The saline pretreated group showed a significant increase in dopamine during the first 20 min of immobilization compared to baseline (one-way ANOVA, $F(8,59) = 3.06$, $P < 0.01$; Fig. 3). In contrast, extracellular dopamine concentrations in the MDMA treated group were unaffected by stress and did not change across time ($F(8,46) = 0.66$).
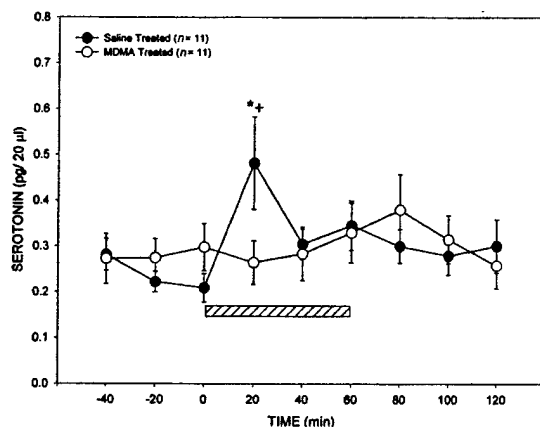

Fig. 2. Effects of 1-h immobilization stress on extracellular levels of serotonin in the prefrontal cortex of rats pretreated 7 days earlier with MDMA (10 mg/kg i.p. for four injections, every 2 h) or an equivalent volume of saline. Baseline microdialysis samples were collected for 1 h, after which rats were immobilized (hatched bar) for 1 h followed by a 1-h post-stress interval. Saline pretreated rats showed an increase in 5-HT concentrations during the first period of immobilization stress (time = 20 min). No change in 5-HT was observed in MDMA pretreated rats ($*P < 0.05$ versus respective MDMA pretreated group; $+P < 0.05$ versus time = 0 of saline treated group).

Fig. 3. Effects of 1-h immobilization stress on extracellular levels of dopamine in the prefrontal cortex of rats pretreated 7 days earlier with MDMA (10 mg/kg i.p. for four injections, every 2 h) or an equivalent volume of saline. Baseline microdialysis samples were collected for 1 h, after which rats were immobilized (hatched bar) for 1 h followed by a 1-h post-stress interval. Saline pretreated rats showed an increase in dopamine concentrations during the first period of immobilization stress (time = 20 min). No change in dopamine was observed in MDMA pretreated rats ($+P < 0.05$ versus time = 0 of saline pretreated group).

Basal serotonin and dopamine levels following MDMA or saline pretreatment

Extracellular concentrations of 5-HT in the dorsal hippocampus were significantly lower in rats treated with MDMA compared to saline treated rats during baseline (0.21 ± 0.02 pg/20 μl vs. 0.38 ± 0.02 pg/20 μl; $t(47) = 4.91$, $P < 0.001$). There were no differences in basal concentrations of dopamine or 5-HT in the prefrontal cortex between MDMA and saline pretreated rats.

Corticosterone and hyperthermic responses to stress

No significant differences in plasma corticosterone concentrations were observed between the saline and MDMA treated rats, either prior to or 10 min into the immobilization stress procedure ($F(1,20) = 0.80$). Immobilization stress increased plasma corticosterone levels in both groups ($F(1,20) = 23.41$, $P < 0.001$, Table 2). There was no difference in peak rectal temperatures during the immobilization procedure between the two groups: saline treated 38.50 ± 0.16, MDMA treated $38.3 \pm 0.22°C$ ($t(9) = 0.04$).

DISCUSSION

MDMA pretreatment attenuates stress-induced neurotransmitter release

The primary, novel finding of the current study is that MDMA pretreatment can inhibit acute 5-HT and dopamine responses to a behavioral challenge, i.e. immobilization stress. The attenuated 5-HT responsiveness is consistent with previous studies using less subtle pharmacological challenges that activate striatal and cortical 5-HT systems (Series et al., 1994; Shankaran and Gudelsky, 1999). A similar attenuation of 5-HT release following a pharmacological challenge has also been reported following other neurochemical lesions of 5-HT neurons, e.g. 5,7-DHT, p-chloramphetamine, or fenfluramine (Baumann et al., 1998; Kirby et al., 1995; Romero et al., 1998; Sabol et al., 1992; Series et al., 1994).

To our knowledge, this is the first report that depleting 5-HT with a serotonergic neurotoxin leads to an attenuation of extracellular dopamine concentrations in response to an environmental challenge. Other reports have suggested 5-HT depletions may augment the responsiveness of dopamine neurons to dopaminergic agents. In MDMA- or 5,7-DHT-induced 5-HT lesioned rats, a challenge injection of cocaine enhanced extracel-

lular levels of dopamine in the nucleus accumbens, increased dopamine metabolism in the prefrontal cortex, and increased conditioned place preference (Horan et al., 1997, 2000; Morrow and Roth, 1996). Restraint stress also increased dopamine metabolism to a greater extent in rats with 5,7-DHT lesions compared to sham controls (Morrow and Roth, 1996). The assessment of dopaminergic activity with ex vivo tissue concentrations rather than *in vivo* microdialysis may explain the discrepancies between Morrow and Roth (1996) and the present findings. Of more interest is the possibility that the toxicity of MDMA to the serotonergic system produces a unique response profile with regard to its longer-term impact on dopaminergic activity.

The blunted stress-induced dopamine response observed in our study may be due to the attenuated stress-induced release of 5-HT in the prefrontal cortex. Increasing 5-HT levels through blockade of 5-HT uptake with fluoxetine or the perfusion of 5-HT itself through a microdialysis probe in the prefrontal cortex increased dopamine levels (Iyer and Bradberry, 1996; Matsumoto et al., 1999). Alternatively, the removal of the normal 5-HT mediated inhibition of GABAergic tone on dopamine cell bodies of the ventral tegmental area (VTA) (Johnson et al., 1992; Kalivas, 1993) may explain the inhibition of stress-induced cortical dopamine release after neurotoxic doses of MDMA. The VTA contains 5-HT fibers, which in part, originate from the dorsal raphe nucleus, a region targeted by MDMA (Herve et al., 1987; Imai et al., 1986; Vertes, 1991). If 5-HT release in the VTA is attenuated following MDMA, GABAergic tone would be enhanced and terminal dopamine release inhibited.

MDMA pretreatment affects basal serotonin levels in the dorsal hippocampus

In addition to the effects on stimulated 5-HT release, basal 5-HT concentrations were lower in rats pretreated with MDMA in the dorsal hippocampus, but not in the prefrontal cortex. The magnitude of 5-HT depletion produced by MDMA may contribute to these regional differences in basal 5-HT concentrations 1 week after MDMA. Decreased basal 5-hydroxyindoleacetic acid levels but not 5-HT concentrations were reported in rats treated with high doses of MDMA in the ventral hippocampus, prefrontal cortex or striatum (Gartside et al., 1996; Series et al., 1994; Shankaran and Gudelsky, 1999). It is possible that a 40–50% decrease in tissue 5-HT as observed previously in the striatum (Shankaran and Gudelsky, 1999) or frontal cortex (Table 2) is not

Table 2. Corticosterone concentrations (μg/dl) in trunk blood plasma collected 7 days following treatment with MDMA (10 mg/kg ip. ×4 injections, every 2 h) or an equivalent volume of saline

Time of corticosterone measurement	Saline treated	MDMA treated
Prior to restraint stress	3.0 ± 2.2	4.5 ± 4.4
During exposure to restraint stress	$20.3 \pm 4.1*$	$20.6 \pm 3.2*$

All data are presented as the mean $\pm$ S.E.M. $*P < 0.05$ vs. prior to restraint stress by a two-way ANOVA.

sufficient to produce changes in basal extracellular concentrations of 5-HT.

A similar relationship between the severity of tissue depletion and the extracellular basal concentrations has been observed following other 5-HT neurotoxic regimens and other monoamine neurotoxins, such as 6-hydroxydopamine. As reported for hippocampal 5-HT in the present study, depletions of tissue norepinephrine content greater than 50% seem to be required for alterations in basal hippocampal norepinephrine (Abercrombie and Zigmond, 1989). In the striatum, Hall et al. (1999) found decreased basal extracellular 5-HT levels following 65% or greater depletions of striatal 5-HT tissue content following 5,7-DHT lesions. However, another study reported no changes in extracellular levels of 5-HT after depletions of 76–93% in striatal 5-HT (Kirby et al., 1995). This later study measured 5-HT levels in anesthetized rats 3 h following probe implantation, rather than in freely moving, awake animals 18 h after probe insertion. Therefore, a longer period for stabilization of extracellular 5-HT concentrations, such as used in this study, may be necessary to measure subtle differences in basal extracellular concentrations of neurotransmitters (Hall et al., 1999).

MDMA pretreatment does not affect stress-induced endocrine or autonomic responses

Central serotonergic systems can modulate stress-stimulated activity of the hypothalamic–pituitary–adrenal axis (for review see Chaouloff, 1993). MDMA, possibly through its ability to increase extracellular 5-HT levels, acutely increases plasma corticosterone (Aguirre et al., 1997; McNamara et al., 1995; Nash et al., 1988). However, these effects of MDMA on corticosterone are not persistent. There were no differences in basal or stress-stimulated corticosterone responses 7 days following MDMA pretreatment (Table 2; Aguirre et al., 1997). This lack of a long-term effect of MDMA on corticosterone responsiveness may be due to the regional selectivity of MDMA-induced 5-HT depletions. MDMA more severely damages 5-HT axons in cortical and limbic terminal regions than in hypothalamic regions (for review see Sprague et al., 1998; Battaglia et al., 1987; O'Hearn et al., 1988; Stone et al., 1986; however see Callahan et al., 2001). Previous studies have reported variable effects of 5-HT lesions on corticosterone levels depending on the neurotoxin used to produce the lesion and the type of stimulus (Baumann et al., 1998; Chung

et al., 1999; Poland, 1990). These differences in corticosterone responses may also be due to the varying degrees of 5-HT depletions in hypothalamic nuclei observed in the studies (Baumann et al., 1998; Chung et al., 1999).

Acute stressors have been shown to produce hyperthermic responses (Kluger et al., 1987; Long et al., 1990; Morimoto et al., 1991; Terlouw et al., 1996). In this experiment, the stress-induced rise in core body temperature occurred in both saline and MDMA pretreated groups. These findings corroborate previous reports that basal, acute stress- or drug-stimulated thermic responses remained intact following 5-HT depletions (McNamara et al., 1995; Chung et al., 1999). Thus, while 5-HT depletions with MDMA may alter neurotransmitter responses to an acute stressor, the autonomic responses appear to remain intact.

OVERALL CONCLUSIONS

Pretreatment with MDMA resulted in 5-HT tissue depletions in the hippocampus and the frontal cortex. MDMA pretreatment blunted the dopamine and 5-HT increases during acute immobilization stress observed in non-depleted or saline pretreated rats. While previous studies have shown that the release of 5-HT in response to a pharmacological agent may be impaired by MDMA pretreatment, the present study indicates that the release of neurotransmitters induced by an environmental change (i.e. stress) also is disrupted. The importance of the various neurotransmitter responses that have been correlated with the onset of an acute stressor is unknown. The increases in neurotransmitter release may be specific to a particular stressor, or may be a more general response to any relevant arousing stimuli. Regardless of the specific behavioral significance, MDMA treated rats exhibited abnormal physiological responses that may have significant implications for long-term functional consequences of MDMA abuse as well as the interactions between the serotonergic system and stress.

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ORIGINAL INVESTIGATION

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Long-lasting effects of chronic stress on DOI-induced hyperthermia in male rats

Abstract *Rationale:* Exposure to chronic stress can affect the serotoninergic (5-HT) system and behavioral measures associated with 5-HT. Repeated stress increases 5-HT receptor subtype 2 (5-HT$_2$) mediated behaviors in rodents, such as wet dog shakes and head twitch. *Objectives:* The current study investigated whether exposure to chronic unpredictable stress would augment 5-HT$_{2A/C}$ receptor-mediated hyperthermia. Furthermore, the persistence of these hyperthermic effects was investigated by testing rats up to 60 days after the stress procedure terminated. *Methods:* For 2 or 10 days, rats were either not stressed (controls) or exposed to chronic unpredictable stress, i.e. two stressors per day of the following: cage rotation, cold exposure, swim, restraint, light cycle manipulations, single housing, and food and water deprivation. After the termination of stress (day 3 or 11), the 5-HT$_{2A/C}$ receptor agonist DOI (1.5 mg/kg) or saline, was injected and the rectal temperature of the rats was monitored. In a separate experiment, the 5-HT$_2$ receptor antagonist, LY-53,587, was injected 30 min prior to the injection of DOI or saline. Finally, DOI was injected into rats 8, 30 or 60 days after the 10-day stress procedure ended. *Results:* Rats exposed to 10 days, but not 2 days, of unpredictable stress exhibited higher rectal temperatures following DOI than non-stressed rats. The DOI-induced hyperthermia was attenuated by LY-53,587. The augmentation of DOI-induced hyperthermia in stressed rats persisted when examined 8, 30 and 60 days following the stress procedure. *Conclusions:* The enhancement of 5-HT receptor function by chronic stress persists even after the environmental stressor is removed. This lasting increase in 5-HT receptor function may have implications for clinical disorders associated with stress, such as depression or post-traumatic stress disorder.

Keywords Chronic stress · Serotonin · 5-HT$_2$ receptor · Hyperthermia

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Introduction

Acute or chronic stress is known to produce alterations in the serotonin (5-HT) neurotransmitter system. Specifically, repeated exposure to social or physical stressors increases the density of the 5-HT subtype 2A (5-HT$_{2A}$) receptor in the cortex (Torda et al. 1988; Davis et al. 1995; McKittrick et al. 1995; Takao et al. 1995; Berton et al. 1998; Ossowska et al. 2001). Increases in the density or affinity of the 5-HT$_{2A}$ receptor also have been shown to consequently augment 5-HT-mediated behaviors. Stimulation of the 5-HT$_{2A}$ receptor by the 5-HT$_{2A/C}$ receptor agonist, (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), altered temperature regulation, wet dog shakes and head shakes in rats or mice (Yap and Taylor 1983; Goodwin et al. 1984; Watson and Gorzalka 1990, 1992; Granoff and Ashby 1998; Lin et al. 1998; Salmi and Ahlenius 1998), and exposure to psychosocial stress or repeated electric foot shock further augmented DOI-stimulated wet dog or head shakes (Metz and Heal 1986; Gorzalka et al. 1998).

Chronic, unpredictable stress exposes a rat to different stressors, rather than the same stressor, over a number of days (10–31). Previous papers have suggested that chronic unpredictable stress produces a valid model of human depression due to its similarity in etiology, symptoms and treatment response (Katz et al. 1981;

Willner et al. 1992; Papp et al. 1996). Chronic, unpredictable stress has been shown also to increase the number of cortical 5-HT$_{2A}$ receptors (Papp et al. 1994; Ossowska et al. 2001), but the behavioral relevance of these changes is unknown. Furthermore, few studies have examined the behavioral or pharmacological responsiveness of the 5-HT system following the termination of stress, which may provide insight into the longer-term changes associated with stress-induced illness such as depression.

Since DOI has been shown to increase body temperature in rats (Pranzatelli and Pluchino 1990; Lin et al. 1998; Salmi and Ahlenius 1998), the present study investigated whether chronic unpredictable stress augments DOI-induced hyperthermia. To assess the persistence of behavioral changes associated with the 5-HT$_{2A/C}$ receptor, hyperthermia was assessed for up to 2 months following the termination of the chronic, unpredictable stress procedure in stressed and control rats.

Materials and methods

Animals and stress exposure

Male Sprague-Dawley rats (175–250 g) were purchased from Zivic Miller Laboratories (Allison Park, Pa., USA). Rats were pair housed in 36×26.5×21.5 cm plastic cages with Santi-Chips covering the cage floor in a temperature-controlled room (mean room temperature=22°C). Rat chow (Purina Mills, Inc., Richmond, Ind., USA) and water (tap) were available without restrictions unless specified by the stress protocol. The rats were maintained on a 12/12 h light/dark cycle with lights on at 6 a.m. and off at 6 p.m. All procedures were in adherence to the National Research Council's Guide for the Care and Use of Laboratory Animals (1996) and approved by the local institutional animal care committee.

Rats assigned to the stress condition were exposed to stressors that varied by day and time for either 2 or 10 days (Stein-Behrens et al. 1994; Fitzgerald et al. 1996; Haile et al. 2001). The following procedure was used for rats exposed to 2 days of stress: day 1 10:00 a.m. 3-min swim stress (23°C), and 6:00 p.m. food and water deprivation (14 h); and day 2 6:00 p.m. single housing and lights on overnight (12 h). The following procedure was used for rats exposed to 10 days of stress: day 1 11:00 a.m. 50-min cold room (4°C), and 12:00 p.m. 60-min cage rotation; day 2 1:00 p.m. 4-min swim stress (23°C), and 6:00 p.m. lights on overnight (12 h); day 3 12:00 p.m. 3-h lights off, and 3:00 p.m. 60-min restraint stress (6×21.6 cm; Harvard Apparatus, Inc. Holliston, Mass., USA); day 4 6: 00 p.m. 50-min cage rotation, and food and water deprivation overnight (14-h); day 5 3:00 p.m. 15-min cold room, and 4:00 p.m. single housing overnight; day 6 11:00 a.m. 3-min swim stress, and 3:00 p.m. 2-h lights off; day 7 1:00 p.m. 30-min cage rotation, and 6:00 p.m. 1-h lights on; day 8 10:00 a.m. 20-min cage rotation, and 3:00 p.m. 60-min restraint stress; day 9 10:00 a.m. 3-min swim stress, and 6:00 p.m. food and water deprivation; day 10 6:00 p.m. single housing and lights on overnight. Stressed and non-stressed rats were weighed daily to monitor their overall health.

Drug injections and temperature measurements

On the test day, all rats were moved to an observation room for a period of 4 h to allow for the stabilization of body temperatures. Ambient temperature of the observation room was 26–27°C. Saline (1 ml/kg) or 1.5 mg/kg DOI hydrochloride (Sigma RBI, St Louis, Mo., USA) intraperitoneal (IP) injections were given to non-stressed and stressed rats. Rectal temperatures were measured 30 min before and 30, 45, 60, 75, 90 and 120 min after the systemic injection with a Thermalert TH-8 monitor (Physitemp Instruments, Inc., Clinton, N.J., USA). For the rectal temperatures, each rat was held by the base of the tail and a probe (RET-2) was inserted 4.6 cm past the rectum into the colon for 6–8 s until a rectal temperature was maintained for 3 s. For rats that were tested following 2 days of chronic unpredictable stress, rectal temperatures were taken at 30 min before and 30, 60, 90 and 120 min after the injection of DOI. In a separate experiment, the 5-HT$_2$ receptor antagonist LY-53,857 maleate (2.1 mg/kg; Sigma RBI, St Louis, Mo., USA) or vehicle was injected IP, 30 min prior to the injection of saline or DOI (1.5 mg/kg), immediately following the first rectal temperature. The number of rats used in each group was as follows: pretreated with vehicle and injected with saline, n=14 control (CVS), n=14 stressed (SVS); pretreated with vehicle and injected with DOI, n=21 control (CVD), n=20 stressed (SVD); pretreated with LY-53,857 and injected with saline, n=8 control (CLS), n=8 stressed (SLS); and pretreated with LY-53,857 and injected with DOI, n=7 control (CLD), n=7 stressed (SLD).

Stressed and control rats were injected with 1.5 mg/kg DOI at 8, 30 or 60 days following the chronic stress procedure to assess 5-HT$_{2A/C}$ receptor mediated hyperthermia over time. On these test days, the same procedures were used as described above and all rats received 1.5 mg/kg DOI. Rectal temperature was measured 30 min before and 30, 60, 75, 90 and 120 min after the DOI injection. The number of rats used in each group was as follows: day 8, n=13 stressed, n=14 control; day 30, n=8 stressed, n=9 control; and day 60, n=11 stressed, n=11 control.

Statistics

Body weights of stressed and non-stressed controls were compared using an independent t-test on days 1 and 11. Mixed factorial analysis of variances (ANOVAs) were used to compare pretreatment (stress versus control) and drug injection (Vehicle+DOI, Vehicle+Vehicle, or LY-53,857+Vehicle, versus LY-53,857+DOI) on rectal temperatures across time; and pretreatment and day tested (8, 30 or 60) on rectal temperatures across time. Significant effects and interactions of pretreatment (stress versus control) were further investigated with two-way ANOVAs followed by Tukey post hoc comparisons. All data are summarized by computing the area under the curve (AUC; summation of the rectal temperature taken at each test time point subtracted from the pre-injection temperature). AUC data for Figs 1, 2 and 3 were analyzed with a one-way ANOVA and data for Fig. 4 were analyzed with a two-way ANOVA (group versus day of test).

Results

Effects of DOI immediately following chronic stress

Stressed and non-stressed control rats did not differ in body weight at the start of the stress protocol [stressed: 213.1±2.6 g; controls: 215.2±2.7 g; t(94)=0.24]. However, by the test day (day 11), control rats weighed more than chronically stressed rats [stressed: 274.3±2.7 g; controls: 301.1±2.9 g; t(94)=6.7, P<0.01].

Systemic injection of 1.5 mg/kg DOI significantly increased rectal temperature in both stressed and control rats over time [2 days of stress: F(4,40)=33.5, P<0.01; 10 days of stress: F(6,150)=69.93, P<0.01]. This effect of DOI compared to saline injected rats was confirmed by the AUC data for rats exposed to 10 days of chronic stress [Fig. 1, F(1,18)=25.5 , P<0.01] and non-stressed control rats [F(1,19)=28.6, P<0.01].
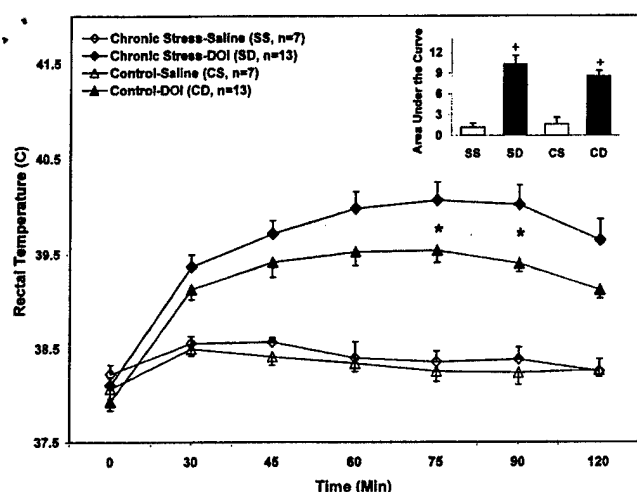
Fig. 1 Injection of 1.5 mg/kg DOI increased rectal temperature of stressed and non-stressed rats relative to basal temperature (time=0) prior to injection (*P*<0.05). Exposure to 10-day stress protocol augmented DOI-induced hyperthermia compared to non-stressed controls when measured 12 h after stress ended (*P<0.05 at 75 and 90 min). *Inset*: comparison of rectal temperatures between groups by AUC (sum of rectal temperature at each time point-baseline temperature). DOI increased rectal temperature in both stressed (*SD*) and control rats (*CD*) compared to saline injected stressed (*SD*) or control (*CD*) rats (+*P*<0.05)
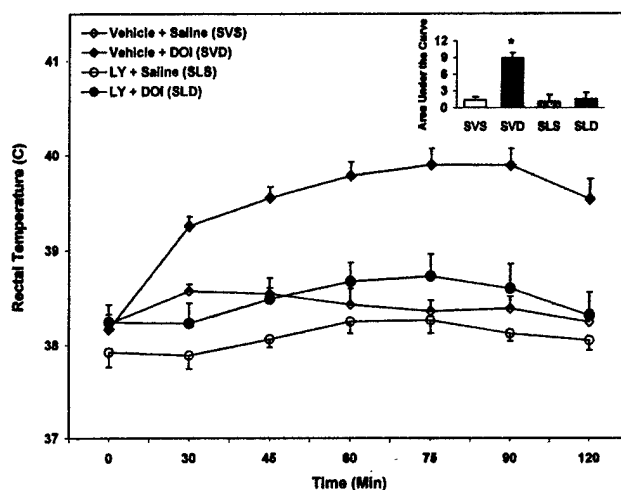
Fig. 2 Injection of 1.5 mg/kg DOI increased rectal temperature of stressed rats relative to basal temperature (time=0) prior to injection (*P*<0.05). Injection of LY-53,857 30 min prior to DOI injection attenuated the DOI-induced hyperthermia. There was no effect of LY-53,857 injection prior to saline or saline injection prior to saline. *Inset*: comparison of rectal temperatures between groups by AUC (sum of rectal temperature at each time point-baseline temperature). DOI increased rectal temperature in stressed rats pretreated with the vehicle (*SVD*) compared to rats pretreated with LY-53,587 (*SLD*) and rats injected with saline and pretreated with vehicle (*SVS*) or LY-53,587 (*SLS*) rats (*P<0.05)

Table 1 Effects of 1.5 mg/kg DOI (IP) on rectal temperature in non-stressed control and stressed rats. Stressed rats (*n*=6) were exposed to 2 days of unpredictable stress, while control rats (*n*=6) were weighed daily. There was no effect of the 2-day stress procedure on DOI-induced hyperthermic response. DOI increased rectal temperature in both control and stressed rats at all time points compared to pre-injection temperatures (time=0)

Time (min)	Non-stressed control	2-Day stress procedure
0	38.03±0.16	38.08±0.15
30	39.17±0.21	39.05±.019
60	39.43±0.27	39.00±0.17
90	39.32±0.29	39.03±0.10
120	38.95±0.22	38.92±0.24

Rats exposed to 10 days of chronic stress had higher rectal temperatures than control rats as indicated by a significant group by time interaction [Fig. 1, $F(6,150)=1.35$, $P<0.05$]. DOI-induced increase in rectal temperature did not differ between rats exposed to 2 days of the stress procedures and non-stressed controls [Table 1, $F(4,40)=1.37$, NS]. Therefore, the 10-day chronic stress procedure was used for subsequent experiments.

The 5-HT$_2$ receptor antagonist LY-53,587 attenuated the DOI-stimulated hyperthermic response of stressed [Fig. 2, $F(18,228)=7.1$, $P<0.01$) and control rats [Fig. 3, $F(18,234)=7.7$, $P<0.01$]. Both stressed and control rats injected with vehicle and DOI had significantly higher rectal temperatures than rats injected with LY-53,587 and saline, or LY-53,857 and DOI [AUC stressed: $F(3,38)=14.6$; AUC control: $F(3,39)=13.7$].
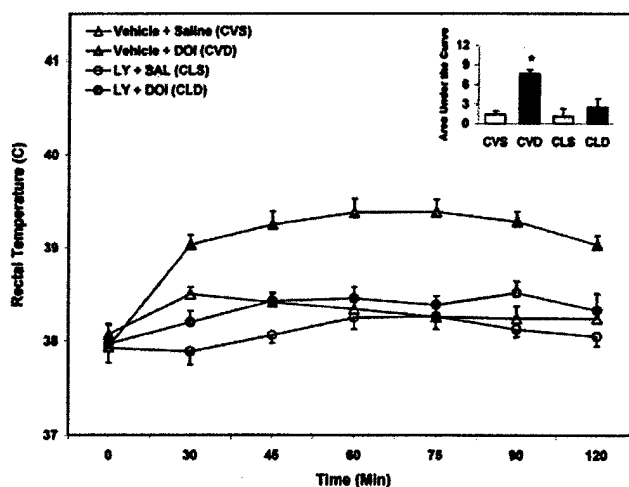

Fig. 3 Injection of 1.5 mg/kg DOI increased rectal temperature of non-stressed rats relative to basal temperature (time=0) prior to injection (*P*<0.05). Injection of LY-53,857 30 min prior to DOI injection attenuated the DOI-induced hyperthermia. There was no effect of LY-53,857 injection prior to saline or saline injection prior to saline. *Inset*: comparison of rectal temperatures between groups by AUC (sum of rectal temperature at each time point-baseline temperature). DOI increased rectal temperature in control rats pretreated with the vehicle (*CVD*) compared to rats pretreated with LY-53,587 (*CLD*) and rats injected with saline and pretreated with vehicle (*CVS*) or LY-53,587 (*CLS*) rats (*P<0.05)
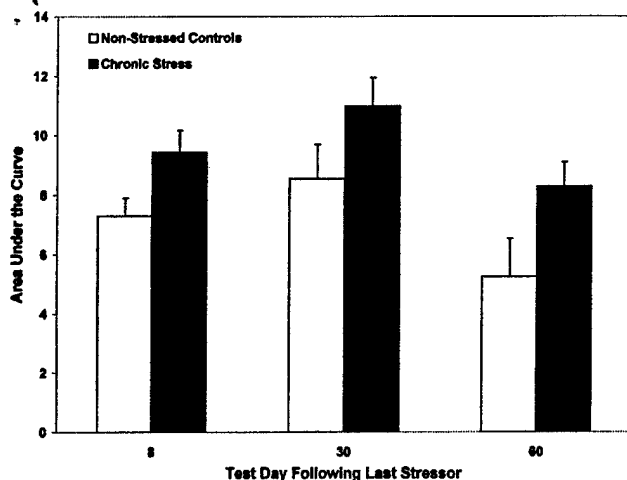
Fig. 4 Exposure to 10-day stress protocol augmented DOI-induced hyperthermia compared to non-stressed controls when measured 8, 30 or 60 days after stress ended ($P<0.05$). The rectal temperature data for five time points are summarized by AUC (sum of rectal temperature at each time point-baseline temperature)

Table 2 Effects of 1.5 mg/kg DOI (IP) on rectal temperature in non-stressed control and chronically stressed rats. DOI was injected 8, 30 or 60 days following the termination of the 10-day chronic stress protocol. Both groups showed a significant increase in rectal temperature at all time points after the drug injection (30, 60, 75, 90, and 120 min) compared to pre-injection rectal temperature (0 min). Rats exposed to 10 days of unpredictable stress showed higher rectal temperatures compared to non-stressed rats

Test day	Time (min)	Non-stressed control	Chronic stress
8 days	0	37.11±0.07	37.07±0.10
	30	38.08±0.11	38.50±0.06*
	60	38.55±0.13	38.92±0.08*
	75	38.69±0.15	39.10±0.10*
	90	38.81±0.19	39.12±0.13*
	120	38.71±0.20	38.91±0.17
30 days	0	37.44±0.22	37.58±0.16
	30	38.76±0.12	38.91±0.15
	60	38.89±0.18	39.56±0.27
	75	39.18±0.19	39.97±0.30*
	90	39.44±0.30	40.18±0.31
	120	39.46±0.46	40.26±0.29
60 days	0	37.58±0.15	37.57±0.19
	30	38.41±0.19	38.84±0.15
	60	38.50±0.22	39.13±0.17*
	75	38.59±0.59	39.26±0.15*
	90	38.77±0.27	39.41±0.17
	120	38.89±0.33	39.51±0.23

*$P<0.05$ compared to non-stressed controls

Effects of DOI 8, 15, 30 or 60 days following chronic stress

Overall, DOI significantly increased body temperature compared to pre-injection rectal temperatures on all test days [$F(5,390)=276.15$, $P<0.01$]. A similar effect was observed when each test day was analyzed separately [day 8: $F(5,125)=209.8$, $P<0.01$; day 30: $F(5,75)=96.7$,

$P<0.01$; day 60: $F(5,100)=64.9$, $P<0.01$]. Stressed rats had significantly higher rectal temperatures compared to non-stressed controls on all test days [Fig. 4, AUC $F(1,60)=11.1$, $P<0.01$]. These differences between stressed and control rats were also evident when each day was analyzed separately [Table 2, day 8: $F(5,125)=3.1$, $P<0.05$; day 30: $F(5,75)=3.1$, $P=0.05$; day 60: $F(5,100)=3.6$, $P<0.05$].

Discussion

Rats exposed to 10, but not 2, days of unpredictable stress exhibited higher rectal temperatures following an injection of the 5-HT$_{2A/C}$ receptor agonist, DOI, than did non-stressed rats (Fig. 1). The DOI-induced hyperthermia was attenuated by the 5-HT$_2$ receptor antagonist LY-53,587, suggesting that the DOI-induced hyperthermia is mediated by 5-HT$_2$ receptors (Figs 2 and 3). Interestingly, the effects of chronic stress on the 5-HT$_{2A/C}$ mediated hyperthermia persisted after the termination of the stress protocol. Increases in rectal temperature following an injection of DOI continued to be greater in chronically stressed rats compared to non-stressed controls when tested 8, 30 and 60 days following the stress procedure (Fig. 4).

Stimulation of 5-HT$_{2A}$ receptors has been shown to increase body temperature in rats (Pranzatelli and Pluchino 1990; Aulakh et al. 1994; Mazzola-Pomietto et al. 1995; Lin et al. 1998; Salmi and Ahlenius 1998). Consistent with the present study, these hyperthermic responses following DOI or other serotonergic drugs were attenuated by pretreatment with 5-HT$_2$ receptor antagonists, such as LY-53,587, ritanserin or ketanserin (Gudelsky et al. 1986; Mazzola-Pomietto et al. 1995; Salmi and Ahlenius 1998; Nisijima et al. 2001). Thus, the current findings and previous studies suggest that DOI stimulates an increase in body temperature through its actions at the 5-HT$_2$ receptor.

In agreement with the present hyperthermia findings, several studies have reported that chronic stress enhances other 5-HT$_{2A/C}$ receptor-mediated behaviors, such as wet dog and head shakes. Head twitch in mice and wet dog shakes in rats were augmented following repeated shock or psychosocial stress (Metz and Heal 1986; Gorzalka et al. 1998). These findings suggest that chronic stress enhances the number or activity of 5-HT$_{2A/C}$ receptors. Metz and Heal (1986) reported an increase in cortical 5-HT$_2$ receptor binding following repeated shock treatment, as their head twitch data would suggest. Other studies have found increases in 5-HT$_{2A}$ receptors following exposure to repeated stress (Torda et al. 1988, 1990; Davis et al. 1995; McKittrick et al. 1995; Takao et al. 1995; Berton et al. 1998; Ossowska et al. 2001). Therefore, an increase in the affinity or density of 5-HT$_{2A/C}$ receptors produced by 10 days of unpredictable stress may account for the potentiated hyperthermic response observed following the injection of DOI.

' The augmented hyperthermia in rats exposed to 10 days of unpredictable stress persisted for 60 days after the stress procedure ended. Given the above discussion, this suggests that stress-induced alterations in the 5-HT$_{2A/C}$ receptor system are long-lasting, even in the absence of additional stressors. Several studies have reported lasting behavioral changes following various chronic or acute stress exposures, such as enhanced fear responses to a novel environment (Desan et al. 1988; Van Dijken et al. 1992a, 1992b; Adamec and Shallow 1993; Pynoos et al. 1996; Koba et al. 2001), escape deficit (Mangiavacchi et al. 2001) or increased self-administration of amphetamine or cocaine (Piazza et al. 1990; Covington and Miczek 2001). To our knowledge, this is the first study that has found a persistent physiological change following exposure to stress that can be attributed to the 5-HT system, specifically.

The brain regions involved in the stress-induced 5-HT$_{2A/C}$ receptor alterations that are responsible for the persistent hyperthermia are unknown; however, an increase in 5-HT$_{2A}$ receptor density in cortex following exposure to stress has been reported (Metz and Heal 1986; Torda et al. 1988, 1990; Davis et al. 1995; McKittrick et al. 1995; Takao et al. 1995; Berton et al. 1998; Ossowska et al. 2001). Alternatively, 5-HT receptors in the hypothalamus are known to mediate temperature regulation (Lin et al. 1983, 1998; Lin and Pivorun 1986) and may also account for the present findings. An injection of DOI (0.2 µg) into the anterior hypothalamus/preoptic area increased colonic temperature, similar to the systemic administration (Lin et al. 1998). Likewise, elevating 5-HT concentrations in the hypothalamus increased colonic temperature, thus, supporting the relationship between 5-HT, the hypothalamus and temperature regulation (Lin et al. 1998).

Several mechanisms may contribute to the 5-HT$_{2A/C}$ receptor alterations following exposure to chronic stress. Chronic unpredictable stress protocols, as used in the current study, have been shown to increase basal or drug-stimulated corticosterone levels in rats (Sapolsky et al. 1984; Haile et al. 2001). Corticosterone, in turn, increases the density of 5-HT$_{2A/C}$ receptors and facilitates 5-HT$_2$ mediated behaviors (Kuroda et al. 1992; McKittrick et al. 1995; Berendesen et al. 1996; Fernandes et al. 1997; Gorzalka et al. 2001). The corticosterone-stimulated increase in 5-HT$_2$ mediated behaviors can be attenuated by the administration of a 5-HT$_{2A}$ receptor antagonist (Gorzalka et al. 2001). It remains to be determined if administration of a 5-HT$_2$ receptor antagonist or a glucocorticoid receptor antagonist during the chronic stress procedure would block the stress-induced hyperthermia.

Alterations in 5-HT transmission have been recorded in many brain regions during and following physical and social stress. Exposure to chronic social stress increases the metabolism of 5-HT in several limbic brain regions, including the preoptic area of the hypothalamus (Blanchard et al. 1991; Berton et al. 1998). Likewise, extracellular levels of 5-HT in the cortex or hypothalamus are elevated during acute immobilization stress (for review see Shimizu et al. 1992; Kawahara et al. 1993; Chaouloff et al. 1999; Matuszewich et al. 2002). Although chronic changes in 5-HT release may alter the density of 5-HT receptors, 5,7-dihydroxytryptamine lesions do not appear to alter the effects of acute immobilization stress on cortical 5-HT$_{2A}$ receptors or 5-HT$_{2A}$ mediated behavior (Torda et al. 1990; Yamada et al. 1995). Despite the fact that acute stress increases 5-HT release, it remains to be determined whether chronic stress produces persistent changes in 5-HT release, which could account for the observed 5-HT-mediated hyperthermic responses. Alternatively, other neurotransmitters or hormones influenced by chronic stress may regulate 5-HT receptor responsiveness (Torda et al. 1990; Gorzalka et al. 2001).

The observed alterations in 5-HT-mediated temperature regulation following chronic, unpredictable stress may be specific to the types of stressors applied or the parameters of the stress exposure. For example, while acute immobilization stress increased extracellular 5-HT in the prefrontal cortex (Kawahara et al. 1993; Matuszewich et al. 2002), forced swim stress for 5 min decreased 5-HT levels (Adell et al. 1997) and did not change 5-HT levels when rats swam for 30 min (Kirby et al. 1995). In the current protocol, a variety of stressors are employed, including swim stress and immobilization. It is not known whether a particular stressor was more critical for changing the 5-HT receptor systems in the current procedure. Sixteen days of exposure to chronic unpredictable stressors, similar to the procedure and types of stressors used in the current study, increased 5-HT$_{2A}$ receptors in the cortex (Ossowska et al. 2001), although the particular stressors used in the protocol have yielded different effects when applied individually (Torda et al. 1988; Chaouloff et al. 1994; Okuyama et al. 1995; Yamada et al. 1995; Durand et al. 1998). In the current study, 2 days of unpredictable stress exposure was insufficient to augment 5-HT$_{2A/C}$-mediated hyperthermic responses (Table 1), suggesting that more than 2 days of unpredictable stressors may be necessary for the 5-HT2A/C mediated behavior changes.

Chronic, unpredictable stress in rodents produces persistent changes in 5-HT$_{2A/C}$ receptor-mediated hyperthermia that last as long as 60 days following the termination of the last stressor. These long-term changes in 5-HT receptor responsiveness parallel the cortical 5-HT$_{2A}$ receptor changes observed in patients with depression (Stahl 1994) and in humans who committed suicide (for review, see Ferrier et al. 1986; McKeith et al. 1987; Arora and Meltzer 1989; Arango et al. 1995; Mann 1998). Thus, the chronic, unpredictable stress model may be useful in identifying neurochemical changes associated with human disorders, such as depression or post-traumatic stress disorder.

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CHRONIC STRESS AUGMENTS THE LONG-TERM AND ACUTE EFFECTS OF METHAMPHETAMINE

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Abstract—There is growing evidence that exposure to stress alters the acute effects of abused drugs on the CNS. However, it is not known whether stress augments the longer-term neurotoxic effects of psychostimulant drugs, such as methamphetamine. Methamphetamine at high doses decreases forebrain dopamine concentrations. The current study tested the hypothesis that 10 days of unpredictable stress augmented striatal dopamine depletions 7 days following four injections of either 7.5 or 10 mg/kg methamphetamine (1 injection every 2 h). Furthermore, to assess the effects of chronic stress on immediate responses to methamphetamine, extracellular striatal dopamine and methamphetamine concentrations, and rectal temperature were monitored during the methamphetamine injection regimen. Seven days following either a 7.5 mg/kg or 10 mg/kg methamphetamine injection regimen, male rats exposed to unpredictable stress showed greater depletions in striatal dopamine tissue content compared with non-stressed controls injected with methamphetamine. Stressed rats had increased hyperthermic responses and dopamine efflux in the striatum during the methamphetamine injections when compared with non-stressed control rats. Moreover, stressed rats had an increased mortality rate (33%) compared with non-stressed controls (16.7%) following four injections of 10 mg/kg methamphetamine. The enhanced acute and longer-term effects of methamphetamine in stressed rats was not due to a greater concentrations of methamphetamine in the striatum, as extracellular levels of methamphetamine during the injection regimen did not differ between the two groups.

In summary, exposure to 10 days of chronic unpredictable stress augments longer-term depletions of dopamine in the striatum, as well as acute methamphetamine-induced hyperthermia and extracellular dopamine levels. These findings suggest that chronic stress increases the responsiveness of the brain to the acute pharmacological effects of methamphetamine and enhances the vulnerability of the brain to the neurotoxic effects of psychostimulants. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: dopamine, hyperthermia, neurotoxicity, psychostimulants, striatum, unpredictable stress.

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Abbreviations: ANOVA, analysis of variance; DAT, dopamine transporter; EDTA, ethylenediaminetetraacetic acid; HPLC-EC, high performance liquid chromatography with electrochemical detection; METH, methamphetamine; PE, polyethylene.

There is increasing evidence that repeated exposure to environmental stressors can alter behavioral and neurochemical responses to drugs of abuse. Prior exposure to stress enhances locomotor activity following a systemic injection of amphetamine, morphine or cocaine (Campbell and Fibiger, 1971; Antelman et al., 1980; Herman et al., 1984; Robinson and Becker, 1986; Kalivas and Stewart, 1991; Deroche et al., 1993; Robinson and Berridge, 1993; Stewart and Badiani, 1993; Deroche et al., 1995). Likewise, drug-induced dopamine release in several forebrain regions is augmented by pre-exposure to stress (Kalivas and Duffy, 1989; Sorg and Kalivas, 1991; Hamamura and Fibiger, 1993; Rouge-Pont et al., 1995). Exposure to stress also facilitates the propensity of rats to self-administer drugs of abuse (for review see Piazza and LeMoal, 1998; Covington and Miczek, 2001) and several studies have reported that prior exposure to methamphetamine (METH) may augment subsequent stress-induced responses in rats and human METH users (Tsuchiya et al., 1996; Yui et al., 1999, 2001; Wallace et al., 2001).

It is unknown if exposure to stress enhances the vulnerability of the dopamine system to the neurotoxic effects of drugs of abuse, in particular, METH. METH can act as a neurotoxin to monoamine neurons when administered at high doses or repeatedly in rodents and non-human primates (Ricaurte and McCann, 1992; Gibb et al., 1993) as evidenced by decreases in the number of tyrosine hydroxylase immunoreactive fibers (Hotchkiss and Gibb, 1980), in the density of dopamine terminals and uptake sites (Bittner et al., 1981; Pu et al., 1994), and in the amount of dopamine in striatal tissue (Ricaurte et al., 1980; Stephans and Yamamoto, 1994). The alterations in these biochemical markers have been reported to endure for months and are most pronounced in the striatum (Seiden et al., 1975; Ricaurte et al., 1980; Bittner et al., 1981; Villemagne et al., 1998). In abstinent human METH users, decreases in the dopamine transporter (DAT) have been reported also in the striata (Volkow et al., 2001). Human drug use is frequently associated with high levels of chronic stress (Koob and LeMoal, 2001); however, it is impossible to assess the relative contribution of stress to the effects of psychostimulant use in humans. The current study will assess whether chronic stress enhances the longer-term damaging effects of METH to the striatum in the rodent model.

Several factors mediate amphetamine-induced neurotoxicity in non-stressed rodents. Hyperthermia, which accompanies high doses of amphetamines, appears to be important for the long-term damage in the striatum. Reducing the core body temperature by pharmacological or environmental manipulations decreases markers of dopa-

mine terminal damage following psychostimulant injections (Ali et al., 1994; Miller and O'Callaghan 1994; Malberg et al., 1996). Another factor that may contribute to the neurotoxic effects of METH is the massive release of dopamine in the striatum during a neurotoxic injection regimen. Blocking METH-induced dopamine release with concurrent administration of dopamine uptake blockers attenuates dopamine depletions, as does inhibiting tyrosine hydroxylase synthesis (Schmidt et al., 1985; Marek et al., 1990; Pu et al., 1994). Chronic stress may influence either or both of these factors to potentiate METH-induced dopamine damage in the brain.

Therefore, the present study investigated the effects of chronic unpredictable stress on the longer-term neurochemical changes in the striatum associated with high doses of METH. We hypothesize that exposure to 10 days of stress will enhance the vulnerability of striatal neurons to METH neurotoxicity as evidenced by greater dopamine depletions. In addition, rectal temperature and acute dopamine release or METH concentrations in the striatum will be monitored during the METH injection regimens to assess any differences between these factors in stressed and non-stressed rats. Previous studies have reported that chronic unpredictable stress augments the motivational and locomotor responses to psychostimulant drugs (Prasad et al., 1998; Haile et al., 2001). The current experiments used the same stress procedure for 10 days, varying the type and timing of different moderate stressors to assess its effects on psychostimulant-induced neurotoxicity.

EXPERIMENTAL PROCEDURES

Animals and stress exposure

Male Sprague–Dawley rats (175–250 g) were purchased from Zivic Miller Laboratories (Allison Park, PA, USA). Rats were pair housed until intracranial surgery with food and water available *ad libitum*, on a 12-h light/dark cycle (lights on at 06:00 h and off at 18:00 h) in a temperature-controlled room (22 °C). All procedures were in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and approved by the local institutional animal care committee. For these studies, efforts were made to minimize the number of rats used and their discomfort throughout the experimental procedures.

Stressed rats were exposed to stressors that varied by day and time for 10 days (Stein-Behrens et al., 1994; Fitzgerald et al., 1996; Haile et al., 2001; Matuszewich and Yamamoto, 2003). The following schedule was followed for rats used in the microdialysis experiments: day 1 11:00 h 50 min cold room (4 °C), and 13:00 h 60 min cage rotation; day 2 13:00 h 4 min swim stress (23 °C), and 18:00 h lights on overnight (12 h); day 3 12:00 h 3 h lights off, and 15:00 h 60 min restraint stress; day 4 18:00 h 50 min cage rotation, and food and water deprivation overnight (14 h); day 5 15:00 h 15 min cold room isolation, and 16:00 h isolation housing overnight; day 6 11:00 h 3 min swim stress, and 15:00 h 60 min restraint stress; day 7 intracranial surgery; day 8 10:00 h 20 min cage rotation, and 15:00 h 2 h lights off; day 9 10:00 h 3 min swim stress, and 18:00 h food and water deprivation overnight; day 10 12:00 h 3 h lights off, and 18:00 h lights on overnight. Unstressed and stressed rats were weighed daily and both groups underwent intracranial surgery on day 7, as listed above.

For the hyperthermia, corticosterone, and ex vivo tissue experiments that did not require intracranial surgery, the same stress procedure was followed until day 6, after which the following schedule was used: day 6 11:00 h 3 min swim stress, and 15:00 h 2 h lights off; day 7 13:00 h 30 min cage rotation, and 18:00 h 1 h lights on; day 8 10:00 h 20 min cage rotation, and 15:00 h 60 min restraint stress; day 9 10:00 h 3 min swim stress, and 18:00 h food and water deprivation; day 10 18:00 h isolation housing and lights on overnight.

Experiment 1: METH treatment of stressed and non-stressed rats

Drug injections and temperature measurements. On day 11, i.p. injections of 7.5 mg/kg or 10 mg/kg d-METH hydrochloride salt (National Institute of Drug Abuse, Bethesda MD, USA), or an equivalent volume of saline (0.9% NaCl), were given every 2 h for a total of four injections. METH was dissolved in saline and given in a volume of 1 ml/kg. The high METH dosing regimen (10 mg/kg×four injections) was selected due to its reliability for causing long-term dopamine depletions in the striatum (Stephans et al., 1998). The lower dosing regimen (7.5 mg/kg×four injections) was selected due to its lower mortality rate. Rectal temperature was measured 30 and 60 min following each i.p. injection with a Thermalert TH-8 monitor (Physitemp Instruments, Inc., Clinton, NJ, USA) by holding each rat at the base of the tail and inserting a probe (RET-2) 4.6 cm past the rectum into the colon for 6–8 s until a rectal temperature was maintained for 3 s. If a rat had a rectal temperature greater than 41.0 °C, wet ice was placed in a tray underneath the cage for 30 min.

Microdialysis procedures. Rats used in the microdialysis experiments were anesthetized with a combination of xylazine (6 mg/kg) and ketamine (70 mg/kg) and placed into a Kopf stereotaxic frame. The skull was exposed and a 21-gauge stainless steel guide cannula (11 mm in length; Small Parts, Inc., Miami Lakes, FL, USA) was positioned above the striatum (+2.0 mm anterior and ±3.2 mm medial to bregma). The cannula and a metal female connector were secured to the skull with three stainless steel screws and cranioplastic cement. An obturator fashioned from 31-gauge stainless steel wire, ending flush with the guide cannula, was inserted into the cannula after surgery.

Four days following surgery, the obturator was removed from the guide cannula and replaced with a microdialysis probe. The microdialysis probes were constructed as previously described (Lowy et al., 1993) from a 27-gauge thin wall stainless steel tube, fitted with a dialysis membrane (13,000 dalton cutoff; 210 μm o.d.; Spectrum Laboratories, Inc., Rancho Domingues, CA, USA) at one end, and a 3 cm piece of polyethylene (PE) 20 tubing (Fisher Scientific, Inc., Pittsburg, PA, USA) at the other end, to serve as the inlet for the perfusion medium. The dialysis membrane was 4 mm×210 μm diameter. A 4 cm length of capillary tubing (125 μm o.d., 50 μm i.d.; Polymicro Technologies, Phoenix, AZ, USA) served as the outlet from the dialysis membrane. The vertical placement of the microdialysis probe was determined during construction of the probe by gluing a ring of PE 20 tubing, which acts as a mechanical "stop," at a measured distance along the length of the probe. The positioning permitted the exposed portion of the dialysis membrane to extend beyond the guide cannula and into the striatum (ventral from dura −1.0 to −5.0). The rats were placed in microdialysis cages and attached via a spring-covered tether to a swivel (Instech Laboratories, Inc., Plymouth Meeting, PA, USA). Dulbecco's phosphate-buffered saline medium (NaCl 138 mM, 2.1 mM KCl, 0.5 mM MgCl$_2$, 1.5 mM KH$_2$PO$_4$, 8.1 mM NaH$_2$PO$_4$, 1.2 mM CaCl$_2$, and 5 mM d-glucose, pH 7.4) was pumped immediately through the microdialysis probe with a Harvard Model 22 syringe infusion pump (Hollison, MA, USA) at a rate of 2.0 μl/min. After a 3 h equilibration period, the following 1 h samples were collected: two baseline samples and eight samples during systemic METH injections.

High performance liquid chromatograph. Microdialysis samples from the striatum were assayed for dopamine by high performance liquid chromatography with electrochemical detection (HPLC-EC). For catecholamine detection, samples (22 μl) were loaded via a Rheodyne injector (Cotati, CA, USA) onto a 3 μ C18 column (100×2 mm; Phenomenex, Torrance, CA, USA). The mobile phase (pH 4.2) consisted of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM EDTA, 0.215 mM octyl sodium sulfate and 3% methanol. Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), with a 6 mm glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were collected using a Hewlett Packard Integrator (Palo Alto, CA, USA).

To assess METH concentrations in dialysate, 40 μl of sample was loaded via a Rheodyne injector onto a 5 μ C18 column (150×2 mm; Phenomenex). A Hewlett Packard 1050 pump delivered 0.455 ml/min of mobile phase (50 mM NaH_2PO_4, 6% acetonitrile, pH=4.6) to the column. Striatal concentrations of METH were detected with a Waters 486 Tunable Absorbance Detector (Milford, MA, USA), with the wavelength set at 245 nm.

Analysis of dopamine tissue concentrations in striatal tissue was performed on an HPLC-EC, as described above. The tissues were sonicated in 0.1 M perchloric acid, centrifuged at 13,000×*g* for 6 min and the supernatant assayed for dopamine. The pellet was re-suspended in 1 N NaOH and protein content determined by a Bradford protein assay.

Histology

One week after systemic METH or saline injections, rats were killed and the brains were quickly removed from the skull and frozen on dry ice (Stephans and Yamamoto, 1994). Probe placements were verified from frozen coronal sections and only rats with probes located in the striatum were used for statistical analysis. Striatal tissue was dissected from a 400 μm slice. The tissue was frozen at −80 °C for later analysis of neurotransmitter content.

Experiment 2: Endocrine measures of stress exposure

On the 11th day, trunk blood and adrenal glands were collected from stressed and non-stressed rats that did not have microdialysis cannula implanted or injections of either saline or METH. All rats were rapidly decapitated between 10:00 and 11:00 h. Trunk blood was collected into a 15 ml vial with 0.3 ml heparin sodium sulfate (1000 U/ml), centrifuged for 15 min (4000×*g*) and the plasma frozen until assayed. From the same rats, adrenal glands were collected and weighed immediately. Corticosterone was measured in 5 μl plasma samples that were diluted with 100 μl of sterile water. The radioimmunoassay employed [125I]corticosterone from ICN Pharmaceuticals (Costa Mesa, CA, USA) and antisera from Ventrex (Portland, ME, USA; Matuszewich et al., 2002). The intra- and inter-assay coefficients of variation were less than 10% with a detectable assay range of 0.1–400 μg corticosterone/100 ml plasma.

Statistical analysis

Independent *t*-tests were used to compare body weight differences (weight on day 11−weight on day 1), corticosterone concentrations, adrenal weights and baseline concentrations of dopamine from chronically stressed rats and non-stressed controls. Adrenal weights were standardized to 100 g of body weight. Two-way, repeated measures analyses of variance (ANOVA) were computed to compare dialysate dopamine or METH concentrations or rectal temperatures over time. Dopamine concentration in dialysate was compared in 10 samples (two predrug samples and eight samples during systemic administration of METH). METH concentration in dialysate

Table 1. Effect of chronic stress on the body weight, adrenal weight and corticosterone levels[a]

	Non-stressed rats	Chronically stressed rats
Body weight (g) (difference day 11-day 1)	71.42±3.67	39.51±3.26*
Adrenal weight (mg/100 g body weight)	11.35±.99	13.55±.73+
Corticosterone (trunk blood μg/dl)	0.89±.60	0.79±.41

[a] All data are presented as the mean+S.E.M. * $P<.01$ when compared to non-stressed rats by an independent Student's *t*-test. + $P<.10$ when compared to non-stressed rats by an independent Student's *t*-test.

was compared in nine samples (one predrug sample and eight samples during systemic administration of METH) because there was no detectable level of METH in the striatum prior to drug injection. Rectal temperature was compared at nine time points (30 min before the first drug injection and 30 and 60 min after each systemic administration of METH). Dopamine tissue concentrations between chronically stressed rats and non-stressed controls were compared in a two-way ANOVA. Post hoc Tukey's pairwise tests were used to analyze any significant treatments. Statistical significance was fixed at $P<0.05$ for all tests.

RESULTS

Effects of chronic stress on body weight, adrenal weight and basal corticosterone levels

Across all experiments, rats exposed to the 10 day stress protocol ($n=49$) showed lower total body weight gain compared with non-stressed controls ($n=47$; $t(94)=6.549$, $P<0.01$; Table 1). There was no statistical difference between stressed and non-stressed adrenal gland weights ($t(18)=1.78$, $P<0.10$) or basal plasma corticosterone levels ($t(18)=0.145$; Table 1). However, of the 10 stressed and 10 non-stressed rats, corticosterone levels were only detectable in four plasma samples of each group.

Effects of chronic stress on METH-induced mortality and hyperthermia

Of the rats exposed to chronic stress, eight of 24 rats that received four METH injections of 10 mg/kg died (33.3%) compared with four of 24 of the non-stressed controls (16.7%). Two of 10 chronically stressed rats died following 7.5 mg/kg METH (20%), while one of 10 non-stressed control rats died (8.3%). None of the rats injected with saline from either group died.

Both stressed and non-stressed rats showed overall increases in rectal temperature after METH injections (7.5 mg/kg: $F(8,128)=8.81$, $P<0.05$; 10 mg/kg: $F(8,56)=13.79$, $P<0.05$). However, chronically stressed rats showed higher rectal temperatures during either 7.5 or 10 mg/kg METH injection regimens compared with non-stressed controls (7.5 mg/kg: $F(8,128)=2.51$, $P<0.05$, Fig. 1a; 10 mg/kg: $F(8,56)=4.56$, $P<0.05$, Fig. 1b). The rectal temperature of stressed rats peaked at 39.2 °C compared with 38.5 °C for non-stressed rats during the four injections of 7.5 mg/kg METH. Likewise, during injections of 10 mg/kg METH, peak rectal temperature of stressed rats reached 40.6 °C while control non-stressed rats reached 39.6 °C.
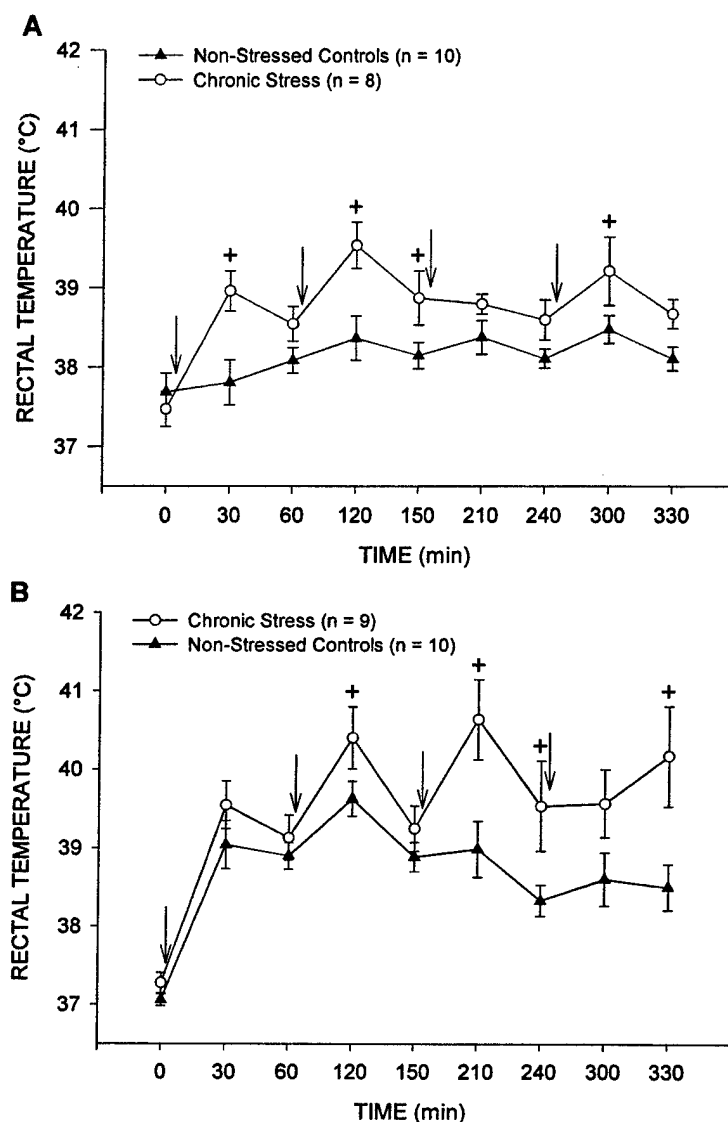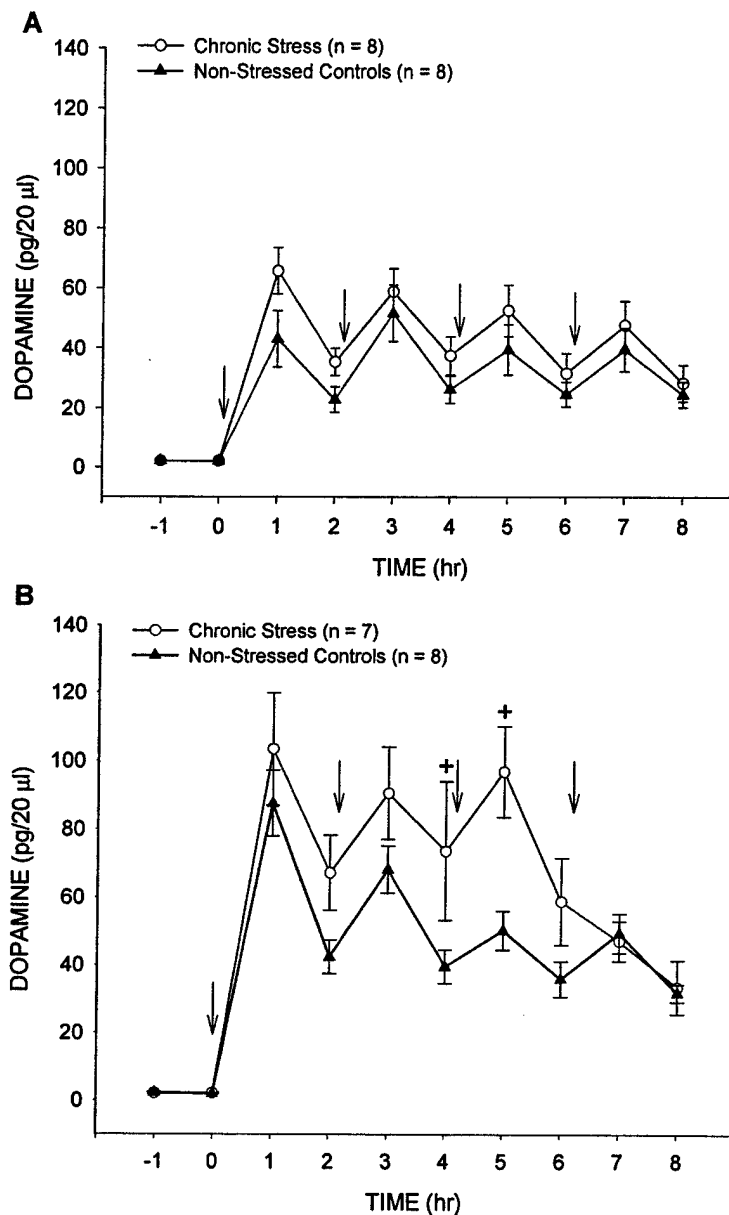
Fig. 1. Rectal temperature increased during injections of (a) 7.5 mg/kg or (b) 10 mg/kg METH (i.p., every 2 h for a total of four injections) compared with temperatures prior to the 1st injection (time=0). Rats exposed to chronic unpredictable stress showed greater hyperthermic responses compared with non-stressed controls ([+] $P<0.05$). Values are expressed as means±S.E.M. Arrow indicates each injection of METH.

Effects of chronic stress on dopamine in the striatum

Extracellular levels of dopamine increased after injections of 7.5 or 10 mg/kg METH (7.5 mg/kg: $F_{(9,126)}=49.9$, $P<0.05$, Fig. 2a; 10 mg/kg: $F_{(9,90)}=35.02$, $P<0.05$, Fig. 2b). Peak dopamine content in dialysis samples was dose-dependent with dopamine concentrations peaking at 66 pg/20 μl following 7.5 mg/kg×four METH, and 110 pg/20 μl following 10 mg/kg×four METH. Chronically stressed rats injected with 10 mg/kg METH showed greater dopamine increases compared with non-stressed controls ($F_{(1,9)}=2.78$, $P<0.05$; Fig. 2b). There was no difference in basal concentrations of dopamine in striatum between stressed (2.08±0.16 pg/20 μl) and non-stressed rats (2.07+0.11 pg/20 μl; $t_{(56)}=0.02$, n.s.).

Prior exposure to stress potentiated METH-induced dopamine decreases in striatal tissue collected 7 days after 7.5 or 10 mg/kg METH. Dopamine concentrations in the striatum did not differ between stressed or non-stressed rats following saline injections ($t_{(28)}=0.23$; stressed: 145.5±14.9 ng/mg protein; non-stressed: 141.1±11.8 ng/mg protein). Therefore, dopamine content is presented graphically as a percentage of the dopamine content in saline-injected striata. Four injections of 7.5 mg/kg METH decreased dopamine content of striatal tissue in stressed rats compared with non-stressed rats injected with METH ($F_{(1,35)}=6.09$, $P<0.05$, Fig. 3a). Both non-stressed and stressed rats had lower striatal dopamine levels following four injections of 10 mg/kg METH relative to saline-injected rats ($F_{(1,31)}=44.54$, $P<0.05$).

Fig. 2. *In vivo* extracellular dopamine concentrations in the striatum prior to and during injections of (a) 7.5 mg/kg or (b) 10 mg/kg METH (i.p., every 2 h for a total of four injections). METH increased dopamine levels for stressed and non-stressed rats relative to baseline concentrations. Rats exposed to chronic unpredictable stress showed greater increases of extracellular dopamine compared with non-stressed controls ([+] $P<0.05$). Values are expressed as means±S.E.M. Arrow indicates each injection of METH.

Dopamine concentrations in the striatum of stressed rats were significantly decreased compared with non-stressed rats treated with four injections of 10 mg/kg METH ($F(1,31)=4.195$, $P<0.05$, Fig. 3b).

Effects of chronic stress on METH concentrations in the striatum

The concentration of METH in the striatum increased following the four injections of 10 mg/kg METH in both stressed and non-stressed rats ($F(8,104)=15.65$, $P<0.01$; Fig. 4), peaking after the 3rd METH injection. However, there were no signif-

icant differences in the extracellular concentrations of METH during the injection regimen between stressed and non-stressed rats ($F(1,13)=0.11$, n.s.).

DISCUSSION

Chronic unpredictable stress augmented the neurotoxic effects of repeated METH injections. One week after administration of METH, decreases in striatal dopamine content were potentiated in rats exposed to chronic stress compared with non-stressed control rats. Systemic injec-
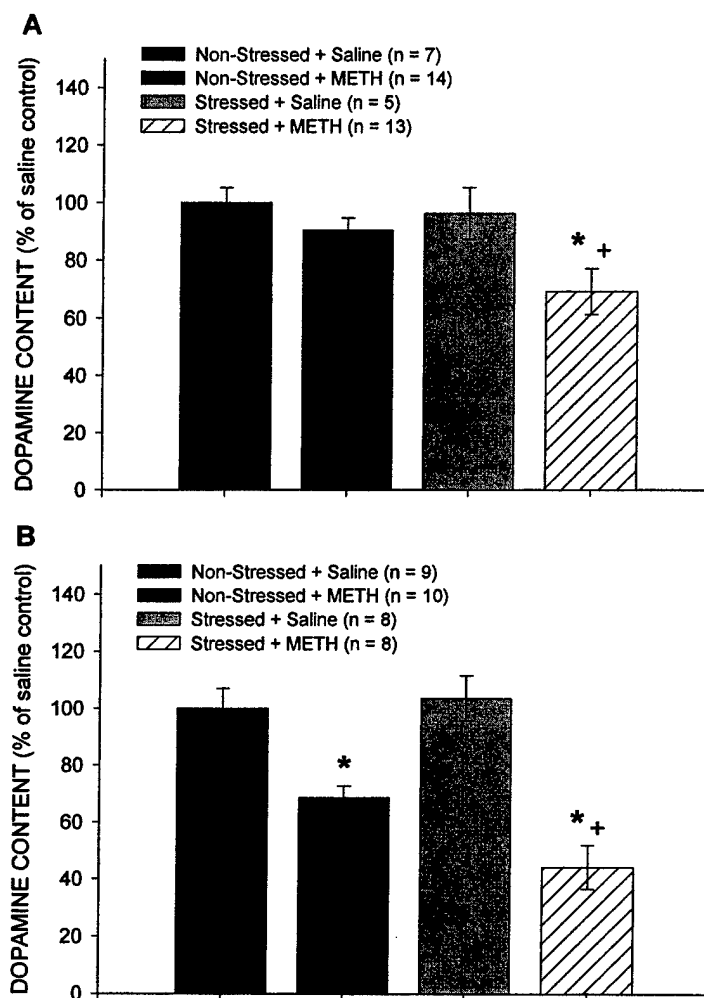
Fig. 3. Ex vivo dopamine concentrations in the striatum 7 days following injections of (a) 7.5 mg/kg or (b) 10 mg/kg METH (i.p., every 2 h for a total of four injections). Injections of 10 mg/kg METH depleted dopamine levels in the striatum for stressed and non-stressed rats relative to rats treated with saline (* $P<0.05$). Rats exposed to chronic unpredictable stress showed greater decrease of striatal dopamine content compared with non-stressed controls following 7.5 or 10 mg/kg METH (+ $P<0.05$). Values are expressed as means±S.E.M.

tions of METH also elevated extracellular levels of dopamine in the striatum to a greater extent in chronically stressed rats than in non-stressed controls. Previous studies in our and other laboratories have shown acute increases in extracellular dopamine during METH administration and subsequent long-term depletions of striatal dopamine (Seiden et al., 1975; Ricaurte et al., 1980; Stephans and Yamamoto, 1994). However, to our knowledge, this is the first report that prior exposure to stress augments the depletion of striatal dopamine content 1 week after drug treatment, as well as the acute release of dopamine during high doses of METH.

Several factors proposed to mediate dopamine depletions following high doses of METH may be responsible for the observed potentiated depletions of dopamine in chronically stressed rats. A long-term depletion of dopamine content in the striatum after METH is correlated with elevated body temperatures during METH administration (Itoh et al., 1986; Bowyer et al., 1994). Pharmacological agents

that lower body temperature attenuate METH-induced dopamine depletions in the striatum, as do lower ambient temperatures (Sonsalla et al., 1991; Bowyer et al., 1992, 1994). In the present study, stressed rats showed greater hyperthermia during METH administration (Fig. 1) and this increase in rectal temperature may contribute to the enhanced dopamine damage observed 1 week after METH injections (Fig. 3).

The increased hyperthermic response also may account for the elevated mortality of stressed rats observed during and following the 10 mg/kg METH injections. Thirty-three percent of the rats exposed to unpredictable stress died compared with 16.7% of the non-stressed rats injected with 10 mg/kg METH, even though cooling was used for both groups to decrease mortality. These results are consistent with the finding that hyperthermia mediates the lethal effects of amphetamines in rodents (Askew, 1962). Central 5-HT receptors contribute to temperature regulation in rats (Salmi and Ahlenius, 1998) and are in-
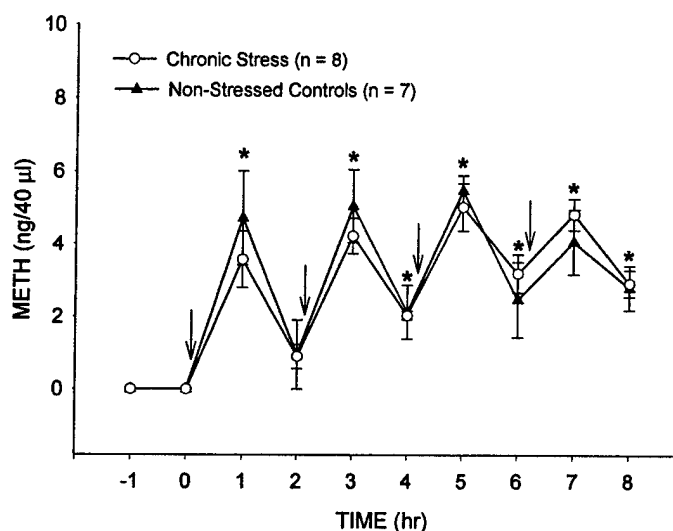
Fig. 4. In vivo extracellular METH concentrations in the striatum prior to and during injections of 10 mg/kg METH (i.p., every 2 h for a total of four injections). Striatal levels of METH increased for stressed and non-stressed rats relative to baseline concentrations (time=0; * $P<0.05$). There was no difference in METH concentrations between rats exposed to chronic unpredictable stress and non-stressed controls. Values are expressed as means±S.E.M. Arrow indicates each injection of METH.

creased following exposure to acute or chronic stressors (Papp et al., 1994; Ossowska et al., 2001). Following the 10-day unpredictable stress paradigm used in the current experiments, stressed rats showed a greater increase in rectal temperature after a systemic injection of a 5-HT$_2$ receptor agonist compared with non-stressed rats (Matuszewich and Yamamoto, 2003). Although stimulation of 5-HT$_2$ receptors may mediate the acute hyperthermia associated with METH, the relationship between 5-HT$_2$ receptor activation and longer-term neurotransmitter content changes after METH remains to be established. Pretreatment with the 5-HT$_2$ receptor antagonist ritanserin failed to prevent METH-induced decreases in tyrosine and tryptophan hydroxylase activities in the neostriatum, 1–20 h after the last METH injection (Johnson et al., 1988, 1994). Furthermore, although chronic treatment with desipramine has been shown to decrease 5-HT$_2$ receptor number or binding density in humans or rodents (Mason et al., 1993; Goodnough and Baker, 1994; Yatham et al., 1999), it did not reduce METH-induced dopamine depletions in either stressed or non-stressed rats (Matuszewich and Yamamoto, unpublished observations).

Alternatively, chronic stress may enhance METH-induced dopamine depletions in the striatum by increasing the acute release of dopamine. Extracellular dopamine concentrations were greater following METH injections in rats exposed to unpredictable stress, than in non-stressed controls (Fig. 2). Blocking dopamine transmission through inhibition of synthesis, blockade of transporter-mediated uptake or co-administration of dopamine antagonists attenuates METH-induced dopamine depletions (Buening and Gibb, 1974; Schmidt et al., 1985; Sonsalla et al., 1986; Marek et al., 1990; Pu et al., 1994). The acute increase in extracellular dopamine may contribute to longer-term dopamine depletions through the generation of free radical

species and quinones (Cubells et al., 1994; Hirata et al., 1995; Huang et al., 1997; Yamamoto and Zhu, 1998; Fumagalli et al., 1999; Larsen et al., 2002).

The potentiation of METH-induced extracellular dopamine levels in rats exposed to stress parallels other findings following a challenge injection of amphetamine or cocaine (for review see Kalivas and Stewart, 1991). Repeated exposure to stress may contribute to the enhanced dopamine release by increasing tyrosine hydroxylase and/or the releasable stores of dopamine, inhibiting dopamine catabolism, decreasing dopamine uptake, or increasing impulse generation in dopaminergic neurons. Ortiz and colleagues (1996) reported an increase of tyrosine hydroxylase in the ventral tegmental area, but not the substantia nigra, following the same 10-day stress procedure as used for the current study. Although increases in dopamine synthesis may explain elevated extracellular dopamine concentrations in the mesolimbic terminal regions, such as the nucleus accumbens, other mechanisms may be operative in the nigrostriatal system (Beitner-Johnson et al., 1991, 1992; Beitner-Johnson and Nestler, 1991; Sorg and Kalivas, 1991).

Due to the ability of METH to release dopamine through reverse transport (Fischer and Cho, 1979), stress-induced alterations in the DAT may account for the augmented release of dopamine during METH injections. However, acute social stress in mice housed in isolation reduced DAT binding in the striatum (Isovich et al., 2001), as did exposure of male tree shrews to chronic subordinate stress (Isovich et al., 2001). These studies suggest that the observed increase in striatal extracellular dopamine in chronically stressed rats is not due to increases in DAT. Interestingly, elevated body temperature also can influence the function of DAT by increasing the intracellular accumulation of METH (Metzger et al., 2000; Xie et al.,

2000). Therefore, the augmented hyperthermic responses in the stressed rats (Fig. 1) may enhance the function of DAT and subsequently contribute to observed increases in extracellular dopamine during METH treatment, irrespective of the number of transporters.

The acute increases or delayed depletions of dopamine observed in the rats exposed to chronic stress do not appear to be due to increased bioavailability of METH in the striatum. METH concentrations in the striatum of stressed and non-stressed rats were similar throughout the METH injection regimen (Fig. 4). While METH concentrations measured with *in vivo* microdialysis suggest that the extracellular concentrations are similar between stressed and non-stressed rats, this technique does not assess the concentrations of METH in dopamine terminals. The concentration of METH in the terminals and subsequent alterations of vesicular pH gradients may be more critical to the longer-term dopamine depletions than extracellular METH concentrations (Sulzer and Rayport, 1990).

Overall, several mechanisms may contribute to the acute increases in hyperthermia or extracellular dopamine in the striatum and the potentiated decreases in dopamine tissue content. The precise effects of repeated, unpredictable stress on the brain are unknown but alterations in 5-HT receptors (Ossowska et al., 2001) or the dopaminergic system (Ortiz et al., 1996; Ossowska et al., 2001), may account for the enhanced hyperthermia, mortality, extracellular dopamine concentrations, or depletions of dopamine content in the striatum. Due to the broad overlap of stress and drug use (Piazza and LeMoal, 1998; Yui et al., 1999, 2001; Koob and LeMoal, 2001), the increased vulnerability of the brain by exposure to unpredictable stressors may be important for understanding the potential detrimental effects of drugs of abuse.

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Effects of amphetamines on mitochondrial function: role of free radicals and oxidative stress

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Abstract

Amphetamine-like psychostimulants are associated with long-term decreases in markers for monoaminergic neurons, suggesting neuronal loss and/or damage within the brain. This long-term "toxicity" results from formation of free radicals, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS), although the mechanism(s) of ROS and RNS formation are unclear.

Mitochondria are a major source of ROS and mitochondrial dysfunction has been linked to some neurodegenerative disorders. Amphetamines also inhibit mitochondrial function, although the mechanism involved in the inhibition is uncertain. This review coordinates findings on the multiple pathways for ROS and RNS and describes a hypothesis involving mitochondrial inhibition in the initiation of amphetamine-induced cellular necrosis.
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Keywords: Amphetamine; Mitochondria; Free radicals; Oxidative stress; Reactive oxygen species; Reactive nitrogen species

Abbreviations: ETC, electron transport chain; NO, nitric oxide; NOS, nitric oxide synthase; ONOO⁻, peroxynitrite; PTP, permeability transition pore; RNS, reactive nitrogen species; ROS, reactive oxygen species; UCP, uncoupling proteins.

Contents

1. Introduction

Amphetamine-like psychostimulants are abused worldwide, and addiction to these drugs poses enormous social and economic burdens. Use of these agents is associated with numerous medical and psychological problems including cardiovascular abnormalities and psychosis. Of particular concern are the long-term striatal dopaminergic and serotonergic deficits associated with repeated exposure to these agents over time. Specifically, studies in rodents and primates have demonstrated long-term deficits in numerous measurements of dopaminergic and serotonergic functions including monoamine transporters, tyrosine and tryptophan hydroxylase, and nerve terminal degradation (Hotchkiss et al., 1979; Wagner et al., 1980; Ricaurte et al., 1982, 1984; Axt & Molliver, 1991; Frey et al., 1997; Villemagne et al., 1998). Although the deficits associated with long-term use of

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amphetamines have been demonstrated, the factors that contribute to these deficits have not been clearly elucidated. Recent data strongly implicate the formation of highly reactive and toxic free radicals, particularly reactive oxygen species (ROS), as a causative factor. Specifically, high-dose administration of amphetamine and amphetamine analogues increases free radicals (Giovanni et al., 1995; Colado et al., 1997; Fleckenstein et al., 1997; Yamamoto & Zhu, 1998) and pretreatment with antioxidants attenuates the amphetamine-induced dopaminergic deficits (Wagner et al., 1985; De Vito & Wagner, 1989; Colado & Green, 1995; Colado et al., 1997; Shankaran et al., 2001). Overexpression of the free radical scavenger superoxide dismutase blocks methamphetamine-induced neuronal deficits (Hirata et al., 1996). Collectively, these studies implicate the generation of ROS in mediating the toxic effects of amphetamine administration.

Numerous sources of ROS can be "activated" by the amphetamines. Under basal physiological conditions, mito-chondria are the *primary* source of intracellular ROS (Chance et al., 1979). The generation of ROS from mito-chondria originates from the complexes in the electron transport chain (ETC) located on the inner mitochondrial membrane and from monoamine oxidase on the outer mitochondrial membrane (Fig. 1A). Amphetamine may enhance the formation of ROS by promoting these basal mitochondrial functions. Since amphetamines cause the release of dopamine, another potential source of these ROS is from the autoxidation of dopamine via the Fenton reaction that uses iron as a cofactor (Graham, 1978). It also has been speculated that increases in intracellular dopamine could lead to formation of reactive dopamine quinones (Graham

et al., 1978), which can in turn generate ROS. Finally, elevation of intracellular calcium and activation of nitric oxide (NO) synthase (NOS) increases the production of highly reactive NO and other reactive nitrogen species (RNS) such as peroxynitrite (ONOO $^-$). The purpose of this review is to present a model and discuss the mechanisms by which mitochondria, in coordination with ROS and RNS generation, produce amphetamine toxicity.

2. Mitochondria and reactive oxygen species production

Mitochondria are the primary source of cellular ATP. This ATP is generated via an ATP synthase that utilizes an inner mitochondrial membrane pH gradient as the driving force. The pH gradient is created by separation of protons (in the form of hydronium ions) and electrons from various donors. The free hydronium ions are used to generate the pH gradient and the free electrons are passed down a series of enzyme complexes (I–IV), known as the ETC, terminating at the final electron acceptor, which is molecular oxygen. This reduction of molecular oxygen forms superoxide ($O_2^-\cdot$) ions that can react with H^+ to form hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) (Fig. 1B). Therefore, a side effect of ATP generation from mitochondria is the formation of ROS, which, under physiological conditions, are "detoxified" by antioxidant systems.

Amphetamines have been demonstrated to inhibit com-plexes within the ETC (Burrows et al., 2000a). This inhibi-tion is likely to increase ROS and contribute to amphet-amine-induced toxicities. There are several reports that

A.

Outer membrane Inner membrane

ETC

Matrix

Innermitochondrial space

B.   Inner mitochondrial space

H^+ H^+ H^+

H^+ H^+

Substrate
(H^+/e^-)

e^- e^-
e^- e^- e^- e^-
e^- e^-

O_2 OH·

H_2O H_2O_2

O_2^-
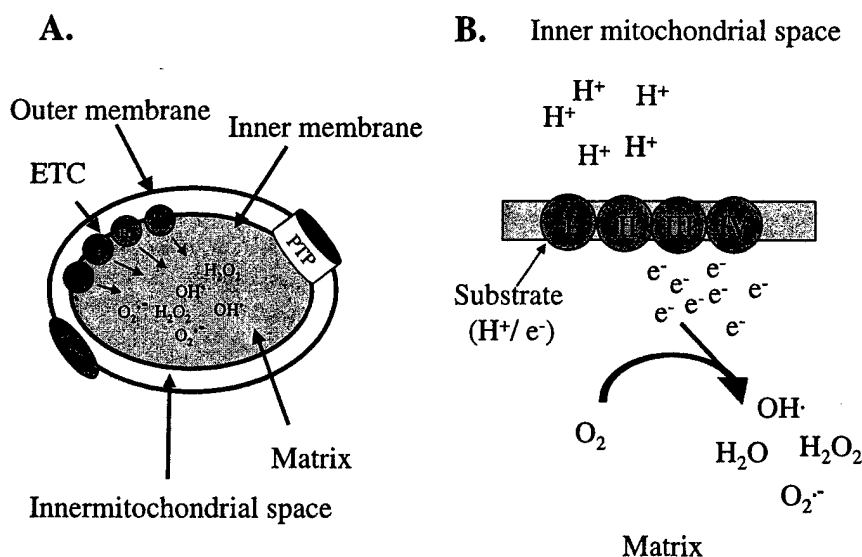
Matrix

Fig. 1. (A) Schematic diagram of mitochondria. Complexes in the electron transport chain (ETC) increase ROS (hydrogen peroxide H_2O_2; superoxide dismutase O_2^-; hydroxyl radicals OH·) in the matrix of the mitochondria. ROS normally are detoxified by antioxidant enzymes (i.e., superoxide dismutase and catalase). The permeability transition pore (PTP) connects the inner and outer mitochondrial membranes, allowing for the passage of molecules between the cytoplasmic compartment and the mitochondrial matrix. Monoamine oxidase (MAO) is responsible for the breakdown of dopamine and can increase H_2O_2 production. (B) The four complexes of the ETC chain (I–IV) remove hydronium ions and electrons from substrates, creating an inner mitochondrial membrane pH gradient. This drives ATP synthase (not shown) and the transference of free electrons to molecular oxygen.

various inhibitors of the ETC will increase ROS formation. For example, the metabolite 1-methyl-4-phenylpyridinium (MPP+) of the dopamine neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an inhibitor of complex I of the ETC (Nicklas et al., 1985) and increases superoxide radical formation (Hasegawa et al., 1990; Thomas et al., 2000). Treatment with Rotenone, another complex I inhibitor, produces long-term damage to dopaminergic terminals in the striatum (Ferrante et al., 1997). Treatment with the neurotoxin 3-nitropropionic acid (3-NPA, a complex II–III inhibitor) also increases ROS production (Schulz et al., 1996). These data suggest that inhibition of complexes within the ETC alters the balance between ROS formation and antioxidant systems such that there is a net increase in ROS accumulation that can damage neurons.

The concept that inhibition of mitochondrial function contributes to neuronal cell death is supported by findings in humans affected with various neurodegenerative disorders. For example, complex I deficiencies in patients with Parkinson's disease has been reported (Schapira, 1998; Schapira et al., 1990) and dramatic decreases in complex II–III are observed in the caudate of patients with Huntington's disease (Gu et al., 1996). Overall, these studies suggest that the inhibition of the mitochondrial ETC can lead to an increase in ROS production, which may underlie or contribute to neurodegeneration.

3. Dopamine-mediated free radical generation

Following administration of amphetamines, particularly methamphetamine, dopamine may accumulate within intracellular compartments of the dopaminergic neurons. This accumulation likely results from a rapid and prolonged inhibition of vesicular dopamine sequestration (Brown et al., 2000). Agents that inhibit the dopamine transporter (and therefore increase vesicular sequestration of dopamine) (Brown et al., 2001) protect against methamphetamine toxicity (Schmidt & Gibb, 1985; Marek et al., 1990). As stated in Section 1, dopamine can autoxidize and form quinones and ROS. Previous studies have demonstrated increased binding of dopamine quinones to cysteine residues on proteins following high-dose methamphetamine administration (LaVoie & Hastings, 1999) and suggest that dopamine autoxidation can form reactive quinones that attack and potentially inhibit the function of intracellular proteins. In addition to dopamine autoxidation, metabolism of dopamine by monoamine oxidase can increase H_2O_2 production and iron-dependent ROS production. For example, inhibition of vesicular sequestration of dopamine with reserpine increases cytosolic concentrations of dopamine and the amount of oxidized glutathione in brain (Spina & Cohen, 1989). This effect is blocked by addition of the monoamine oxidase inhibitor, clorgyline (Spina & Cohen, 1989), suggesting that metabolism of dopamine by monoamine oxidase increases ROS production.

Monoamine oxidase-mediated dopamine metabolism and subsequent H_2O_2 formation also can inhibit mitochondrial ETC activity. For example, Berman and Hastings (1999) demonstrated that dopamine inhibits complex II–III of the ETC via monoamine oxidase-induced formation of H_2O_2. In a separate study, it was demonstrated that dopamine inhibited complex I, an effect that was attenuated by the iron chelator desferrioxamine (Ben-Shachar et al., 1995). Interestingly, desferrioxamine also attenuates the long-term depletion of dopamine after methamphetamine (Yamamoto & Zhu, 1998). These reports support a feed-forward model of dopamine-mediated toxicity involving increases in intracellular ROS formation that results from elevated intracellular dopamine and dopamine metabolism, which in turn inhibits the ETC to further increase ROS formation and subsequent toxicity.

Yuan et al. (2001) place less importance on the role of dopamine in mediating the toxic effects of high-dose methamphetamine treatment. They observed that when body temperature changes are minimized depletion of dopamine with reserpine and/or α-methyl-p-tyrosine did not attenuate the deficits induced by methamphetamine administration (Yuan et al., 2001). Further studies by this group revealed that the toxic effects of MDMA are similarly independent of dopamine (Yuan et al., 2002). Additionally, not all brain regions rich in dopamine are damaged by methamphetamine. For example, the medial prefrontal cortex is relatively spared from the long-term deficits induced by methamphetamine treatment (Eisch et al., 1992; Broening et al., 1997). Likewise, not all regions damaged by amphetamines contain dopamine. Studies have demonstrated damage induced by methamphetamine to the hippocampus, a region largely devoid of dopamine (Brunswick et al., 1992). Finally, if intracellular dopamine accumulation is critical to amphetamine toxicity, amphetamines should also damage noradrenergic neurons, given that fact that dopamine is the precursor of norepinephrine and the synthesis of norepinephrine occurs only after dopamine has been sequestered into synaptic vesicles. Numerous studies, however, have demonstrated that high-dose methamphetamine administration does not produce long-term deficits in markers for adrenergic neurons (Wagner et al., 1980; Ricaurte et al., 1984; Preston et al., 1985). These observations taken together argue that additional factors and/or transmitters (e.g., glutamate) may be responsible for increasing ROS production.

4. Glutamate-mediated reactive nitrogen species generation

High-dose methamphetamine treatments increase glutamate release in the striatum (Nash & Yamamoto, 1992; Abekawa et al., 1994; Stephans & Yamamoto, 1994) that can activate glutamate receptors on dopamine terminals (Page et al., 2001; Tarazi & Baldessarini, 1999). Other studies have linked both ionotropic and metabotropic glu-

tamate receptors with the dopaminergic toxicity associated with administration of amphetamines. Sonsalla et al. (1989) demonstrated that NMDA (ionotropic) receptor antagonists prevent neurotoxicity induced by methamphetamine. One confound to these studies, however, is that these agents, particularly MK-801, prevent the methamphetamine-induced hyperthermia (Ali et al., 1996), which may account for the neuroprotection. However, this does not preclude the possibility that NMDA receptors contribute to the neurotoxic effects of amphetamines. Metabotropic glutamate receptors may also mediate the toxic effects of amphetamines. For example, mGluR5 receptor blockade prevents methamphetamine-induced deficits in a temperature-independent manner (Battaglia et al., 2002).

Activation of glutamate receptors, particularly NMDA receptors, leads to massive calcium influx and mitochondrial disruption as well as increased superoxide formation (Schinder et al., 1996; Prehn, 1998). This statement is supported by the finding that glutamate infused directly into the striatum increases 2,3-dihydroxybenzoic acid (2,3-DHBA) formation (indicative of OH formation), an effect that is blocked by the NMDA receptor antagonist MK-801 and by addition of the antioxidant α-phenyl-N-tert-butyl nitrone (Lancelot et al., 1998). Therefore, glutamate receptor activation on dopamine terminals increases calcium influx and generation of free radicals within dopamine neurons.

Increased calcium influx can also activate NOS and increase the formation of reactive NO, which in turn can inhibit mitochondrial function. Studies in hippocampal neuronal cultures demonstrate that NO rapidly depolarizes mitochondrial membranes and produces a progressive depletion of ATP (Brorson et al., 1999). This mitochondrial inhibition is likely due to an irreversible blockade of complex II–IV in the ETC (Bolanos et al., 1994). Moreover, NO-mediated inhibition of mitochondria is dependent on activation of NMDA receptors since the inhibition of complex II–IV of the mitochondrial ETC in rat cortical neurons by glutamate is blocked by L-NAME and APV (NMDA receptor antagonists) (Almeida et al., 1998). Interestingly, neuronal complex II–III can be inhibited by the formation of ONOO$^-$ that is produced by the interaction of H_2O_2 and NO (Bolanos et al., 1995). Similar phenomena can occur within dopamine terminals in the striatum. H_2O_2 is formed from metabolism of dopamine by monoamine oxidase and/or the oxidation of dopamine (Graham, 1978), whereas NO is produced from glutamate receptor activation and calcium influx. The hypothesis that amphetamine toxicity is linked to the formation of ONOO$^-$ is supported by the finding that glutamate-mediated increases in NO and ONOO$^-$ as well as 3-nitrotyrosine (3-NT), a marker of ONOO$^-$ production, are increased in PC12 cells and the rat striatum after methamphetamine treatment (Imam et al., 2001). Treatment with the NO inhibitors S-methylthiocitrulline, 3-bromo-7-nitroindazole, or 7-nitroindazole prevents the methamphetamine-induced decreases in dopamine, its metabolites, and dopamine transporter binding (Itzhak & Ali, 1996; Itzhak et al., 2000).

Therefore, these data suggest a role for ONOO$^-$ in mediating the inhibition of mitochondrial ETC activity and subsequent dopamine toxicity.

5. Amphetamine-induced hyperthermia and reactive oxygen species production

A sustained elevation of core body temperature produced by methamphetamine has been linked directly to the dopaminergic deficits produced by the drug (Bowyer et al., 1994). Hyperthermia may contribute to the increase ROS production since prevention of methamphetamine-induced hyperthermia attenuates the formation of ROS in the striatum (Fleckenstein et al., 1997). The ETC is susceptible to elevated temperatures (Skonieczna et al., 1986) and Lepock et al. (1987) demonstrated that O_2 consumption (an indirect measure of ETC function) in hamster V79 cells increases linearly from 8 to 40 °C whereas above 40 °C O_2 consumption is strongly inhibited. These data are suggestive of the possibility that elevated body temperatures (i.e., >40 °C, as seen with high-dose methamphetamine administration) can alter ETC function and increase ROS formation. Therefore, amphetamine-induced hyperthermia may enhance ROS formation by decreasing mitochondrial ETC activity. Additionally, hyperthermia increases the utilization of cellular ATP (Madl & Allen, 1995), which may contribute to the necrotic cell death associated with amphetamines (see Section 6).

Of potential relevance to methamphetamine-induced hyperthermia are mitochondrial uncoupling proteins (UCP). UCP regulates the balance between heat production and energy expenditure in cells (for review, see Argyropoulos & Harper, 2002). UCP can inhibit the mitochondrial ETC and dissipate the energy as heat under circumstances of decreased energy needs. The inhibition of mitochondrial function by UCP may be protective by decreasing the extent of mitochondrial-generated free radicals (Arsenijevic et al., 2000; Echtay et al., 2002). No studies to date have examined the effect of amphetamine administration on UCP function. However, it can be hypothesized that amphetamines may increase ATP utilization and increase ROS production via inhibition of UCP. Conversely, increased UCP activity following administration of amphetamines may be neuroprotective by decreasing ROS production. Clearly, further studies of UCP following high-dose amphetamine administration are warranted.

6. Amphetamine, ATP, and mechanisms of cell death

Previous studies have suggested that amphetamines decrease ATP levels by increasing ATP utilization and inhibiting ATP production. For example, methamphetamine administration decreases ATP levels in the striatum 1.5 and 3 hr after drug administration (Chan et al., 1994; Wan et al., 1999). Enhancement of mitochondrial energy metabolism

through fatty acid oxidation by carnitine protects against 3-NPA-induced neurotoxicity and methamphetamine-induced 3-NT formation (Virmani et al., 2002). Infusions of substrates for the ETC also attenuate methamphetamine-induced neurotoxicity (Stephans et al., 1998). Interestingly, the protection afforded by ETC substrates was only seen when administered 6 hr following the last methamphetamine treatment, suggesting that mitochondrial inhibition occurs hours after methamphetamine is administered (Stephans et al., 1998). In contrast, when the complex II inhibitor, malonate, is combined with the local infusions of methamphetamine or MDMA (local infusion of amphetamines into the brain does not produce the monoaminergic deficits that are associated with their systemic administration), there is a synergistic enhancement of the depletion of dopamine and serotonin content in the striatum (Burrows et al., 2000b; Nixdorf et al., 2001).

In addition to decreased ATP production, amphetamine may increase ATP demand. Hyperthermia and hyperactivity associated with amphetamines is likely to increase ATP utilization and decrease overall energy stores. Along these lines, glycogen phosphorylase utilization is increased following MDMA administration (Poblete & Azmitia, 1995; Darvesh et al., 2002) and overall glycogen levels are rapidly depleted following administration of amphetamines (Huether et al., 1997). Therefore, an amphetamine-induced inhibition of complexes in the ETC and the associated decrease in the production of ATP, coupled to the increase in ATP demand, may rapidly deplete energy stores and result in cell death.

The relationship between decreases in mitochondrial function and the type of cell damage (i.e., necrosis vs. apoptosis) produced by methamphetamine is complex. Necrosis does not affect individual cells but is characterized by general swelling and inflammation of the cell and mitochondria to produce smoothing of the plasma membrane and a disordered structure of the mitochondria. In contrast, apoptosis affects individual cells and is defined by cell shrinkage and fragmentation without inflammation, blebbing of the plasma membrane, increased membrane permeability of the mitochondria even though the mitochondrial structure is relatively preserved, and fragmented as well as clumped nuclei and DNA. The typical neurochemical consequence of high-dose methamphetamine administration is that dopamine terminals in the striatum are lost, whereas the corresponding dopamine cell bodies located in the substantia nigra remain largely unaffected. Ricaurte et al. (1982) demonstrated that high-dose methamphetamine treatment results in granular degradation in the striatum but not in the substantia nigra, thus suggesting local necrosis of dopamine terminal loss. Since individual dopamine cell bodies appear unaffected by methamphetamine administration, it is unlikely that apoptosis, which would destroy soma and eventually the axon terminal, occurs after methamphetamine administration. It is possible that ROS formation and mitochondrial inhibition produced by methamphetamine favor necrosis over apoptosis.

Rotenone is an inhibitor of complex I of the mitochondria, is similar to methamphetamine, and damages dopamine neurons in the striatum via an ROS-mediated mechanism. Although both rotenone and methamphetamine inhibit mitochondrial function and cause cellular damage via ROS, rotenone produces a loss of dopamine cell bodies and terminals (Betarbet et al., 2000), whereas methamphetamine spares dopamine soma while damaging dopamine terminals (Ricaurte et al., 1982). This apparent discrepancy between alterations in mitochondrial function and the type of damage produced by the two drugs could be explained simply by the possibility that necrosis of dopamine terminals after methamphetamine is insufficient to initiate apoptosis in the corresponding soma. Alternatively, the depletion of ATP concentrations by amphetamines may actually block the apoptotic pathway whereas rotenone, at doses that are sufficient to produce apoptotic cell death, inhibits complex I activity without affecting ATP concentrations (Betarbet et al., 2000). Therefore, the presence or absence of ATP depletion, simultaneous with the inhibition of mitochondrial function, may have a significant impact on whether necrosis or apoptosis is produced. Support for this hypothesis comes from Leist et al. (1997) and Qian et al. (1999) who demonstrated that prior depletion of ATP by only 50% could switch the mechanism of cell death from apoptosis to necrosis. In a similar study, depletion of ATP prevented caspase activation and switched the mechanism of cell death from apoptosis to necrosis (Terminella et al., 2002). The mechanisms mediating the switch from apoptosis to necrosis remain to be determined but most likely involve local changes within the mitochondria.

Mitochondria are important regulators of apoptosis in addition to being involved in the generation of ATP. Regulation of apoptosis occurs primarily at the level of the permeability transition pore (PTP) located on the outer membrane of the mitochondria. (Fig. 1A). The opening of the PTP allows for the passage of low molecular weight (<1.5 kDa) compounds between the mitochondria matrix and the cellular cytosolic compartments. Association of the PTP with the proapoptotic factor Bax allows for the passage of cytochrome c (13 kDa) from the inner membrane space of the mitochondria to the cytosolic space (Jurgensmeier et al., 1998). Release of cytochrome c activates a cascade of apoptotic events culminating in the activation of proteases and the consequent degradation of cellular proteins. Since caspase activation is regulated mainly by the release of cytochrome c from the ETC via the association PTP and Bax, prior depletion of ATP likely influences either the opening of the PTP or the association of the PTP with proapoptotic factors such as Bax. Therefore, a rapid and substantial depletion of ATP that precedes PTP opening may explain how amphetamine produces necrosis rather than apoptosis. As discussed in Section 4, methamphetamine also increases NO production. NO-dependent necrosis can result from the inhibition of mitochondrial function and depletion of ATP because when ATP levels are restored or normalized

in the presence of NO, apoptosis occurs (Leist et al., 1999). These results suggest that amphetamine-induced necrosis could be explained by increased NO production with consequent reductions of ETC activity and ATP prior to mitochondrial PTP opening.

Although amphetamines appear to damage striatal dopaminergic terminals via necrosis, there is evidence that neurons within the striatum may undergo apoptosis following administration of amphetamine-like psychostimulants. For example, Deng and Cadet (2000) observed two markers of apoptosis (i.e., activation of caspase-3 and poly(ADP-ribose)polymerase (PARP) cleavage) in mice following methamphetamine treatment. A neurotoxic regimen of methamphetamine in rats also induces long-term decreases (3 months) in striatal preprotachykinin mRNA (found within GABAergic cell bodies) (Johnson-Davis et al., 2002). Additionally, neuronal inclusions, which stain positive for ubiquitin, have been identified in GABAergic projection neurons in mice following MDMA treatment (Fornai et al., 2002). Transmitter phenotype markers for striatal GABAergic and cholinergic neurons, glutamate decarboxylase and choline acetyltransferase, respectively, are not altered by methamphetamine (Hotchkiss et al., 1979). If the latter is taken to indicate that a normal complement of striatal neurons is retained, then it is reasonable to consider that the changes in apoptotic factors reflect neuroadaptations or damage to GABAergic neurons rather than neuronal loss per se. Further

studies are needed to fully understand the potential toxicity to neurons in the striatum.

7. An amphetamine model of dopamine toxicity: inhibition of mitochondrial function

Based on the above overview, the following model of amphetamine toxicity is proposed (refer to Fig. 2). Initial administration of high-dose amphetamines increases intracellular dopamine via inhibition of vesicular sequestration of dopamine (Brown et al., 2000; Hogan et al., 2000). As cytoplasmic dopamine increases, dopamine is rapidly metabolized by monoamine oxidase, resulting in increased production of H_2O_2 and other ROS. Dopamine that is not rapidly metabolized autoxidizes to form reactive quinones that (1) attack cytoplasmic proteins and lipids and (2) increase the formation of ROS. In addition to these immediate effects of intracellular dopamine, extracellular dopamine increases due to an initial efflux of dopamine through the dopamine transporter (Jones et al., 1998). Extracellular dopamine stimulates postsynaptic dopamine receptors, which activate a polysynaptic loop resulting in an increase in cortical glutamate release into the striatum (Stephans & Yamamoto, 1994). Glutamate activates ionotropic and metabotropic glutamate receptors on striatal dopamine terminals to increase calcium influx (Tarazi & Baldessarini, 1999). As
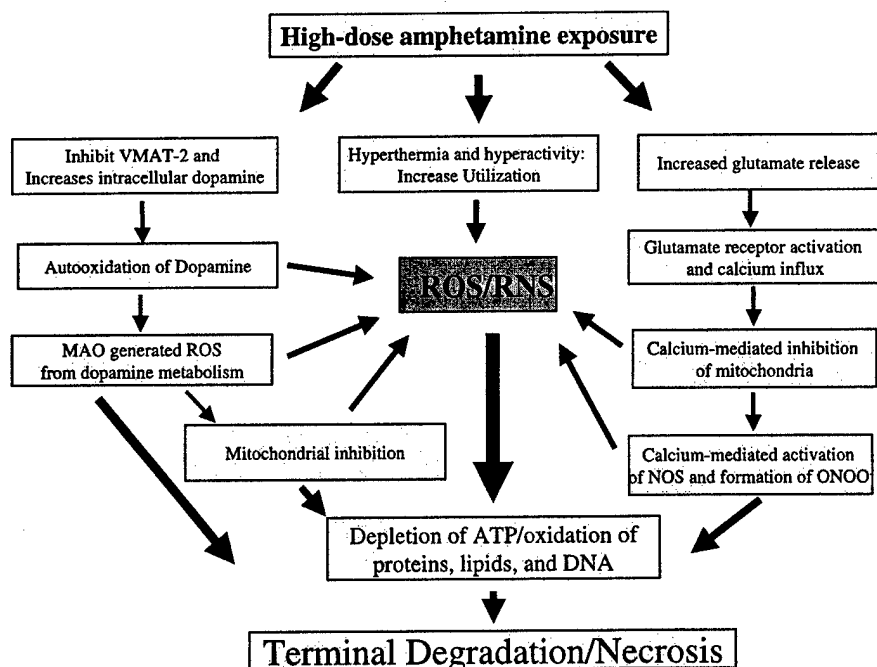
Fig. 2. Proposed hypothesis for amphetamine-induced necrosis. Following exposure to high-dose amphetamine(s), multiple pathways are activated including those underlying hyperthermia as well as striatal increases in presynaptic cytoplasmic dopamine and glutamate release. These pathways increase the burden of ROS/RNS and deplete energy stores, resulting in mitochondrial inhibition. Inhibition of mitochondria by further increasing ROS production, collapsing the pH gradient, and elevating intracellular calcium allows for opening of the PTP and initiating of necrotic pathways (in the absence of ATP). The end result is loss of the associated terminals that is reflected by decreases in monoamine transporters, tyrosine and tryptophan hydroxylase activities, and nerve terminal degradation.

intracellular calcium concentrations increase, mitochondrial function is inhibited and ROS production is increased. Calcium increases NOS activity, which in turn produces NO radicals and $ONOO^-$. This new source of free radical is likely to further inhibit mitochondrial activity, increase ROS and RNS formation, and increase the burden on antioxidant systems. Coupled with these effects is amphetamine-induced hyperthermia, which may enhance the process of ROS formation at all stages and may itself inhibit mitochondrial function. The net outcome of these effects is an increased ROS and RNS production that overwhelms antioxidant systems and initiates apoptotic pathways. However, since activation of apoptosis requires ATP utilization and administration of amphetamine rapidly depletes ATP levels, the mechanism of cell death favors necrosis over apoptosis of dopamine terminals. Blockade of any one of these steps is likely to decrease sufficiently the total free radical load so that antioxidant systems are able to compensate for the increase in ROS production. Therefore, depletion of dopamine, increased vesicular dopamine uptake, blockade of glutamate receptors, inhibition of NOS, or prevention of hyperthermia may be sufficient to decrease the free radical burden such that antioxidant systems can effectively counteract the increased ROS production.

8. Clinical implications

Rodent studies typically employ a neurotoxic regimen of amphetamine administration composed of four injections of 10 mg/kg every 2 hr. This regimen is based on the shorter half-life of amphetamines in rodents versus humans and is hypothesized to model the human "binge" pattern of amphetamine use. In addition, the deficits induced by this dosing regimen to rodents mimic the deficits seen in dopamine transporter density, dopamine content, and tyrosine hydroxylase protein seen in humans (Wilson et al., 1996). As stated above, numerous approaches can be applied prophylactically to prevent or attenuate the toxic effects of amphetamines on humans. However, a therapeutically relevant agent would be effective when administered post-amphetamine exposure. It has been demonstrated that dopamine transporter inhibitors and mitochondrial substrates can reverse the deficits induced by amphetamine when administered after a high-dose regimen (Marek et al., 1990; Stephans et al., 1998). Current treatment for amphetamine overdose includes cooling the patient to combat amphetamine-induced hyperthermia. No retrospective studies have assessed long-term effects of hyperthermia prevention or the usefulness of other neuroprotective agents (i.e., antioxidants, dopamine transporter inhibitors, or mitochondria substrates) in human amphetamine abusers. However, as models of amphetamine toxicity become refined and further mechanisms of toxicity are elucidated, new therapeutic approaches can be tested and may provide benefit against long-term deficits associated with amphetamines.

9. Conclusion

The amphetamine family of psychostimulants can produce long-term deficits in dopaminergic and serotonergic systems in the brain. These "toxicities" likely result from dopamine- and glutamate-generated ROS and RNS that inhibit mitochondrial function to further increase ROS and decrease ATP production. The cumulative effect is loss of associated nerve terminals. Although multiple pathways converge to produce neurodegeneration, intervention at a single pathway to or within the mitochondria is sufficient to prevent or attenuate toxicity. Therefore, multiple targets are available that can drive the design of a potentially large number of beneficial therapeutic agents. More specifically, the development of an understanding of how amphetamines damage neurons and how therapeutic innervations can prevent these deficits may provide for the design of novel drugs that can treat or prevent neurodegeneration.

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A Rapid and Selective Decrease in Mitochondrial Complex II Activity by Methamphetamine

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Abstract:

Methamphetamine (METH) is a psychostimulant of abuse that causes long-term deficits in striatal dopaminergic and possibly GABAergic systems. Although the mechanism(s) which contribute to these deficits are not clearly defined, dopamine and glutamate are likely to play key roles. These factors are hypothesized to converge and may inhibit normal mitochondrial function, increasing the intracellular free radical burden and decreasing neuronal energy supplies. Previous studies suggest a potential role for changes in the mitochondrial electron transport chain (ETC) in contributing to METH-induced neuronal deficits. The purpose of the present studies was to determine if high-dose METH administration selectively inhibits complex II of the ETC. Results demonstrate that METH produced a rapid (within 1 hr) decrease in complex II (succinate dehydrogenase) activity. In addition, decreased activity of complex II-III, but not complex I-III, of the mitochondrial ETC was also observed at 24 hrs after METH. This inhibition was not due to direct inhibition by METH nor dependent on METH-induced hyperthermia. The decreases in complex II-III were associated with striatal, but not hippocampal, brain regions. Decreases in complex II-III were prevented by MK-801 treatment after METH administration. These findings provide the first direct evidence that METH administration, via delayed glutamate receptor activation, selectively alters a specific site of the ETC and implicate mitochondrial inhibition as an early event that occurs after the administration of METH but may be a causative factor to its long-term toxicity.

High-dose methamphetamine (METH) administration results in long-term deficits in monoaminergic systems (Hotchkiss et al., 1979; Ricaurte et al., 1982; Wagner et al., 1980; Axt and Molliver, 1991; Villemagne et al., 1998; McCann et al., 1998; Frey et al., 1997). In addition, emerging evidence suggests that METH may damage non-dopaminergic, particularly GABAergic, neurons in the striatum (Jayanthi et al., 2004). Although these deficits have been described, the mechanism(s) involved in mediating these changes remain largely unknown. However, numerous factors have been implicated, including dopamine and glutamate, which have been hypothesized to converge at a single target, mitochondria, to produce damage to neuronal elements via oxidative stress (for review see Brown and Yamamoto, 2003). Therefore, mitochondrial inhibition may represent the initial catalytic site which mediates these long-term deficits in markers of striatal neurons.

Mitochondria regulate ATP synthesis, apoptotic and possibly necrotic cell death. A key feature of mitochondria is the mitochondrial electron transport chain (ETC), which is responsible for ATP synthesis and a possible site for regulation of cell death. The ETC is a series of four complexes (I-IV) which pass free electrons via oxidative phosphorylation to form ATP. The products of this oxidative phosphorylation include water and numerous free radicals such as hydroxyl and superoxide radicals. In fact, it has been speculated that mitochondria represent the largest source of endogenous free radicals (Chance et al., 1979). Inhibition of normal ETC function, or even specific complexes within the ETC, can increase free radical production. For example, inhibition of complex II with 3-nitropropionic acid (3NP) increases free radical production within neurons of the striatum (Kim et al., 2002).

Recent studies provide suggestive evidence that high-dose METH administration alters complex II within the ETC, an effect that may contribute to the long-term deficits associated with METH. For example, direct infusion of METH into the striatum does not result in long-term depletions of dopamine however, direct infusion of METH with the complex II inhibitor, malonate, results in long-term depletions of striatal dopamine content that were greater than that

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seen with malonate alone (Nixdorf et al., 2001). In addition, the combination of METH with a low dose of 3NP markedly increased the frequency of striatal lesion formation verses 3NP administration alone (Reynolds et al., 1998). These findings are of interest because unlike other complexes in the ETC, little is known about the regulation of complex II by pharmacological agents and in particular METH. The present studies were conducted to directly test the hypothesis that high-dose METH administration rapidly inhibits complex II. Results demonstrate that METH decreases complex II activity in a manner dependent on glutamate receptor activation. These findings provide the first direct evidence that METH administration selectively alters a specific site of the ETC and implicates mitochondrial inhibition as an early event in the toxicities associated with high-dose METH administration.

METHODS:

Animals:

Male Sprague-Dawley rats (250-300 g; Charles River Laboratories) were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled environment. Rats were initially housed two to four per cage in plastic cages with food and water available ad libitum. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Boston University Medical School institutional animal care and use committee.

Treatments:

High-dose METH was administered in a series four single injections (1 injection every two hours) of 10mg/kg s.c. All rats were killed 1 or 24 h following the last drug administration. Vehicle (saline) administrations were given in a corresponding manner as METH at dose of 1ml/kg, s.c. MK-801 (Sigma) was administered 1 hr and 5 hours after the last METH administration. In experiments where METH-induced hyperthermia was prevented, one cage, containing METH-treated animals, was placed on ice during the entire dosing regimen (normothermic and METH-treated). Rectal temperatures were monitored throughout the treatment period.

Preparation of mitochondria:

Striatal and hippocampal tissue was removed and rapidly frozen on dry-ice. No differences in baseline enzyme rates were seen between fresh and previously frozen tissue (data not shown). Once all samples were collected, tissues were homogenized in homogenization buffer (final concentrations: 1% cholate and 30 mM potassium phosphate pH 7.5) at a

concentration of 50 mg/ml (original tissue wet weight) and incubated on ice for 5 mins. The homogenate was centrifuged at 4°C (14,000 x g 5 min) to remove membranous fragments.

Complex I-III/II-III Assay

Activity of mitochondria complex I-III and II-III were assessed as described previously (Brass et al., 2001). An aliquot (50μL) of the homogenate was added to the appropriate incubation buffer. The buffer was prepared fresh for each assay (Final concentrations: EDTA 100μM, BSA 0.2%, and potassium phosphate 50mM pH 7.5 @ 37°C, cytochrome C 150μM, and asolectin 150μg/ml). After preincubation, substrate was added for the measurement of complex I-III, (NADH 2 mM final concentration) or complex II-III (succinate 40 mM final concentration). All assays contained potassium cyanide to inhibit complex IV (1.5mM final concentration). Upon addition of substrate, samples were placed in a Versamax plate reader (Molecular Probes) and reduction of cytochrome C was measured at 550nM for 10 min at 37°C. Because of the poor solubility of ETC inhibitors in aqueous solutions, enzymes rates were calculated by subtraction of activity in the absence of substrate (i.e. succinate or NADH) from substrate dependent activity. No difference was noted in enzyme rates in the presence of inhibitor or in the absence of substrate (data not shown). All samples were normalized to protein concentrations as determined using a commercially available protein assay (Bio-Rad). All samples were run in quadruplicate and average rates calculated.

Succinate Dehydrogenase Assay

Activity of complex II (succinate dehydrogenase) activity was assessed as previously described (Brass et al., 2001). Briefly ~100 ug protein was incubated in Tris buffer (final concentration: 100mM-0.1% Tris-BSA pH 7.8; 0.5mM phenazine sulphate, ~ 1mM DCPIP) and incubated at 37°C for 5 min. The reaction was initiated by the addition of succinate (40 mM

final). Enzyme activity was monitored spectrophotometrically by the reduction of DCPIP/PES at 600nM. Enzyme rates were calculated from the reciprocal of the slope and normalize to protein as described above.

Statistics:

Results were compared using a Students' T-Test, ANOVA followed by a Student-Newman-Kuels post-hoc analysis or a two-way ANOVA as indicated. Significance was set at $p \leq 0.05$.

RESULTS:

To determine if high-dose METH administration alters mitochondrial ETC activity, male Sprague-Dawley rats were treated with high-dose METH and mitochondrial activity was determined 1 h after the last drug administration. Complexes II-III, but not I-III, were rapidly (within 1 hr) inhibited by high-dose METH administration (figure 1). To examine the role of METH-induced hyperthermia in the decrease in complex II-III activity, a third group was included whereby METH-induced hyperthermia was prevented (see methods). Average rectal temperatures for each treated group were as follows: Sal $37.2 \pm 0.3^{\circ}C$, METH hyperthermic $39.8 \pm 0.6^{\circ}C$ ($p < 0.001$ verse SAL), METH normothermic $37.7 \pm 0.7^{\circ}C$ ($p < 0.001$ verse METH alone). Prevention of METH-induced hyperthermia did not reverse the METH-induced decrease in complex II-III activity (figure 2). The inhibition of complex II-III activity is specific for striatal, but not hippocampal, brain regions (figure 3) and is unlikely due to residual METH in the mitochondrial preparation. This latter statement is based on the finding that direct application of METH in concentrations up to ~10 mM was without effect on complex II-III activity (figure 4).

Because decreases were observed in complex II-III but not complex I-III, it was hypothesized that the METH-induced inhibition was specific for complex II. The test this hypothesis, complex II activity (i.e. succinate dehydrogenase) was examined independent of complex I or III. Use of specific complex inhibitors confirmed the selectivity of this assay for complex II (figure 5). Data presented in figure 6 demonstrate that METH inhibits the activity of complex II.

Previous studies examining the effect of METH on mitochondrial function suggest that infusion of mitochondrial substrates hours <u>after</u> the high-dose METH treatment afforded protection against METH-induced dopaminergic deficits (Stephans et al., 1998). In addition high-dose METH causes a delayed raise in extracellular glutamate (Nash and Yamamoto, 1992) which can persist for at least 12 hours after METH administration (unpublished observation).

Previous studies examining the effect of glutamate receptor stimulation demonstrate a specific inhibition of complex II enzyme activity (Dabbeni-Sala et al., 2001). These data suggest that delayed increases in striatal glutamate following METH may contribute to the selective decrease in complex II activity. To assess the role of long-term complex II inhibition and delayed glutamate elevation, complex II-III activity was assessed 24 hours following high-dose METH administration in the presence and absence of the glutamate receptor antagonist MK-801. This time point was selected to represent the latest time point following METH administration in which normal DAT activity is not lost (Metzger et al., 2000) (i.e. no loss of dopamine terminals). Results demonstrate that complex II-III activity (figure 7) was decreases 24 hr following high-dose METH administration as compared to saline controls. No difference in II-III activity was detected with late MK-801 treatment in the presence of METH.

DISCUSSION:

High-dose METH administration rapidly and selectively inhibits the activity of complex II in the mitochondrial ETC (figure 1). This inhibition of complex II activity was specific for striatal, but not hippocampal brain regions (figure 3) and is unrelated to METH-induced hyperthermia (figure 2) or residual METH in the mitochondrial preparation (figure 4).

Several experiments elucidated the mechanism mediating the decrease in complex II activity. The observed effects are not due to a direct effect of METH since incubation of mitochondria with concentrations of METH that are typically achieved in suspensions of striatal tissue (~59nM) from brain after a systemic injection regimen of METH (Haughey et al., 2000) had no effect on complex II-III or I-III activity. Only high micromolar concentrations of METH produced a non-selective inhibition of complexes I-III (data not shown) and II-III (figure 4). In addition, the decrease in complex II-III was not associated with METH-induced hyperthermia since prevention of hyperthermia did not reverse the decrease in complex II-III activity (figure 2). Previous studies showed that prevention of hyperthermia is neuroprotective against METH-induced dopaminergic deficits (Albers and Sonsalla, 1995). However, hyperthermia contributes to, but is not solely responsible for, METH-induced deficits (Albers and Sonsalla, 1995). Thus, the decrease in complex II-III may mediate a hyperthermia-independent component of the METH toxicity to dopaminergic or non-dopaminergic elements. Alternatively, hyperthermia may enhance the toxic effects of mitochondrial inhibition but does not contribute directly to mitochondrial inhibition.

The early decreases (i.e. within 1hr) in complex II activity observed in these studies represent a decrease of ~25-30% at 1 hr. This change may not reflect the actual magnitude of the METH-induced decreases since the mitochondrial preparation utilized in the present studies is from numerous cell types within the striatum. There are no current methods for isolating mitochondria from a specific population of cells. Therefore, the exact location of the affected mitochondria as well as the exact decrease in complex II is difficult to ascertain precisely.

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However, numerous studies support a role of mitochondrial inhibition in mediating the deficits induced by high-dose METH. Agents which support mitochondrial respiration protect against METH-induced dopaminergic deficits (Stephans et al., 1998) such that the infusion of decylubiquinone, which serves as an electron donor downstream of complex II, completely protected against METH-induced deficits only when infused <u>after</u> the METH administrations. These results suggest that a delayed and prolonged inhibition the mitochondrial ETC contributes to the METH-induced dopaminergic deficits. The present study extends this study by demonstrating a prolonged (up to 24 hrs) and selective inhibition of complex II after METH administration. Further support for inhibition of complex II contributing to METH-induced deficits has been provided by Burrows et al., (2000) where intracranial infusion on METH only caused long-term deficits in dopaminergic systems when co-administered with a specific complex II inhibitor malonate. Finally, combination of METH with a low dose of 3NP, a specific complex II inhibitor, markedly increased the frequency of striatal lesion formation verses 3NP administration alone (Reynolds et al., 1998). These data and the present findings strongly implicate the METH-induced inhibition of complex II in the long-term deficits induced by high-dose METH administration.

Although the exact mechanism of this decrease in complex II activity remains to be determined, METH-induced increases in striatal glutamate contribute. Specifically, administration of an NMDA receptor antagonist MK-801, even injected *after* the administration of METH, prevented the METH-induced decrease in activity of complex II-III (figure 7). Dabbeni-Sala et al. (2001) demonstrated that incubation with a glutamate receptor agonist causes a selective loss of the complex II activity. These effects were prevented by a glutamate receptor antagonist and antioxidant treatment (i.e. melatonin or GSH) and point to glutamate-receptor mediated ROS formation directly inhibiting complex II. Moreover, METH produces a delayed increase in striatal glutamate (Nash and Yamamoto, 1992), which can in turn activate glutamatergic ionotropic receptors and damage dopamine terminals in the striatum. This

increased stimulation of glutamate receptors may initiate a feed-forward mechanism that occurs predominately *after* the METH administration regimen and results in inhibition of complex II and the further formation of ROS. The finding that the administration of MK-801 *after* METH can prevent the decrease in complex II activity is consistent with the delayed rise in glutamate (Nash and Yamamoto, 1992) and suggests that late occurring excitotoxic events to complex II can still be blocked pharmacologically. These data may have significant implications for the treatment of METH overdose.

The hypothesis that ROS formation inhibits complex II activity is supported by studies reported by Gluck et al. (2002). Specifically, complex II-mediated cellular respiration is most sensitive to inhibition by dopamine- and monoamine oxidase-mediated hydrogen peroxide formation. Similar results were reported by Berman and Hastings (1999) wherein dopamine-mediated mitochondrial respiration was decreased (~35%) when assessed in the presence of succinate (complex II substrate) while bypassing complex I. These findings support the role of ROS in mediating a selective inhibition of complex II. Interestingly, METH administration has been shown to alter vesicular sequestration of dopamine (Brown et al., 2000; Brown et al., 2002) and may therefore increase dopamine intracellularly which can autoxidize and increase the intracellular ROS burden. Therefore, dopamine oxidation may represent another source of ROS that inhibits complex II following high-dose METH treatment.

A decrease in complex II activity can augment the oxidative burden contributing to neuronal damage. Decreases in mitochondrial respiration can further increase ROS formation (Kim et al., 2002) leading to a self-perpetuating loss of complex II activity and ROS formation. Furthermore, decreases in cellular antioxidant systems such as glutathione after METH have been reported (Harold et al., 2000) and suggest that increased ROS can overwhelm antioxidant defense systems and potentially damage proteins and lipids (Yamamoto and Zhu, 1998).

Inhibition of complex II of the mitochondrial ETC can decrease ATP production. Previous studies show that METH decreases striatal ATP concentrations (Chan et al., 1994).

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These changes in ATP were localized to the striatum and not hippocampal brain regions and are consistent with the selective decrease in complex II in the striatum observed in the present study (figure 3). Because depletion of ATP can favor necrosis (Leist et al., 1999; Terminella et al., 2002) and METH damages dopaminergic terminals without affecting dopaminergic cell bodies in the nigra (Ricaurte et al., 1982) (suggestive of necrosis), inhibition of complex II and decreases in ATP could contribute to the dopaminergic deficits seen after METH administration (Brown and Yamamoto 2003). However, METH can also activate apoptotic factors in the striatum of mice (Deng and Cadet, 2000). *In vitro* METH application using a striatal GABAergic cell line (M213) shows a specific inhibition of complex II mediated respiration and activation of apoptosis (Deng et al., 2002). Since the effect of METH on complex II activity seen in the present study can not be localized to a specific neuronal population, the METH-induced inhibition of complex II may mediate damage to other non-monaminergic neurons. Recently, high-dose METH administration has been shown to decrease GAD67 immunoreactivity (Jayanthi et al., 2004) and substance P (Johnson-Davis et al., 2002) mRNA levels long-term after METH administration. Collectively these studies are suggestive of a mitochondrial mediated toxicity to non-dopaminergic, possibly GABAergic, cells in the striatum (Cadet et al., 2003). Regardless, the present findings are consistent with the proposed scenario of a complex II inhibition, ATP depletion, and increased ROS formation as initial catalytic events which contribute to necrotic and/or activation of apoptotic factors in the striatum produced by METH.

Unlike other ETC complexes, little is known about the regulation of complex II by pharmacological agents. Although agents such as malonate directly inhibit complex II activity, the present study demonstrates that other pharmacological agents such as METH can indirectly, but selectively inhibit this complex through glutamate-mediated mechanisms. This rapid and persistent loss of complex II activity produced by METH may have long-term consequences on neuronal function. Moreover, the scope of this finding may not be limited to METH but may extend to other neurodegenerative disorders. For example, Huntington's disease is a

neurodegenerative disorder whereby GABA containing neurons in the striatum are lost. In fact, patients with Huntington's disease have a persistent deficit in striatal complex II activity (Gu et al., 1996). In addition, inhibition of complex II by 3-NP mimics the neuronal deficits seen in Huntington's disease patients. As stated above, administration of 3NP (specific complex II inhibitor) with a low dose of METH increases the number of striatal lesions when compared to 3-NP alone (Reynolds et al., 1998). Although no direct links between METH abuse and Huntington's disease have been reported, the mechanism of damage may be similar. Clearly, further investigations into the regulation of complex II and its contribution to neurodegenerative disorders are warranted.

In conclusion, these studies provide the first direct evidence that high doses of METH rapidly decrease complex II activity via glutamate receptor, and possibly, ROS-mediated mechanisms. This inhibition may represent the initial catalytic event that occurs predominately after and not during METH administration and contributes possibly to METH-induced toxicities and perhaps other neurodegenerative disorders.

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Figure 1: Effect of high-dose METH administration on mitochondrial ETC activity. Columns represent mean enzyme activity of complex I-III or complex II-III in mitochondria isolated from the striata of vehicle- (1ml/kg/2 hr X 4; solid columns) or METH- (10mg/kg/2 hr X 4; open columns) treated rats. All rats were killed 1 hr following the last injection. Bars represent 1 S.E.M. for eight animals. * Values different from vehicle-treated controls ($p \leq 0.05$) using Student's T-Test.

Figure 2: Role of METH-induced hyperthermia on the decreases in complex II-III activity. Columns represent mean enzyme activity of complex II-III in mitochondria isolated from the striata of vehicle (1ml/kg/2 hr X 4; solid columns) or METH (10mg/kg/2 hr X 4; open columns) treated rats. METH-induced hyperthermia was prevented by placing 1 cage containing METH treated animals on ice (normothermic/METH-treated group). All rats were killed 1 hr following the last injection. Bars represent 1 S.E.M. for eight animals. * Values different from vehicle-treated controls ($p \leq 0.05$) using one-way ANOVA.

Figure 3: Effect of high-dose METH administration on mitochondrial ETC activity in striatal or hippocampal brain regions. Columns represent enzyme activity of complex II-III in mitochondria isolated from the striatum or hippocampus of vehicle- (1ml/kg/2 hr X 4; solid columns) or METH- (4X 10mg/kg/2 hr X 4; open columns) treated rats. All rats were killed 1 hr following the last injection. Bars represent 1 S.E.M. for eight-eleven treated animals. * Values different from vehicle-treated controls ($p \leq 0.05$) using Student's T-Test.

Figure 4: Effect of in vitro application of METH on complex II-III activity. Mitochondria, isolated from the striatum of untreated rats, were incubated with increasing concentration of METH and complex II-III activity was assessed. Points represent the mean enzyme activity and bars represent 1 S.E.M. for three determinations of enzyme activity.

Figure 5: Effect of specific mitochondrial inhibitors: antimycin A (complex III: AA, 5µg/mL), rotenone (complex I: RT, 20mM), 3NP (complex II: NP, 1uM), DMSO (Vehicle: DMSO, 0.3%), or control (Tris Buffer) on complex II activity. Columns represent mean enzyme rate ± 1 S.E.M. * Values differ from all other treated groups ($p \leq 0.05$) using a one-way ANOVA.

Figure 6: Effect of high-dose METH on complex II activity in striatum from treated rats. Animals were treated with either vehicle (1ml/kg/2 hr X 4; solid columns) or METH (10mg/kg/2 hr X 4; open columns). Animals were killed 1 hr after the last injection. Columns represent mean enzyme rate ± 1 S.E.M. * Values differ from vehicle-treated group (p≤0.05) using a Student's T-test.

Figure 7: Role of glutamate receptors in contributing to the METH-induced decreases in complex II activity. Rats were treated with vehicle (1ml/kg/2 hr X 4; solid columns) or METH (10mg/kg/2 hr X 4; open columns) with or without a post-administration of MK-801 (1mg/kg 1 hr and 5 hr following the last vehicle or METH administration). Al animals were killed 24 hours after the last vehicle of METH administration. Columns represent the mean rate of complex II activity (in nmoles/min/μg protein) from the striata of treated animals. Bars represent 1 S.E.M. for eight-nine treated animals. * Values different from corresponding controls (p≤0.05) using a Student's T-Test.

Figure 1

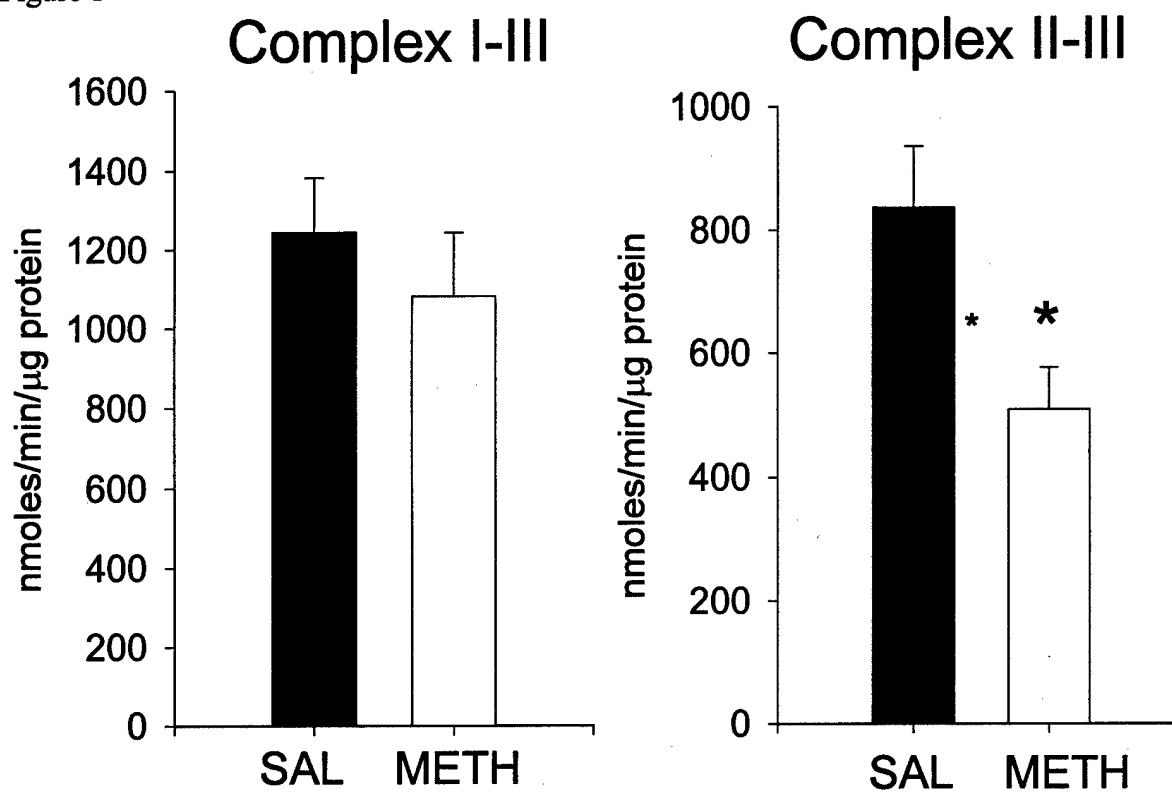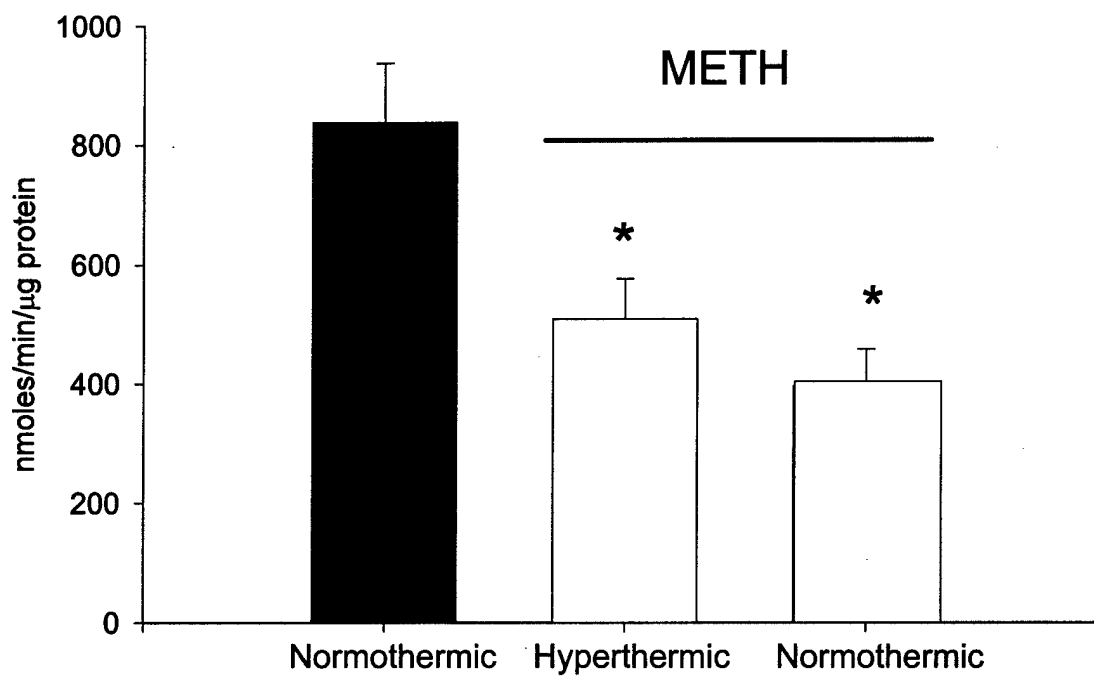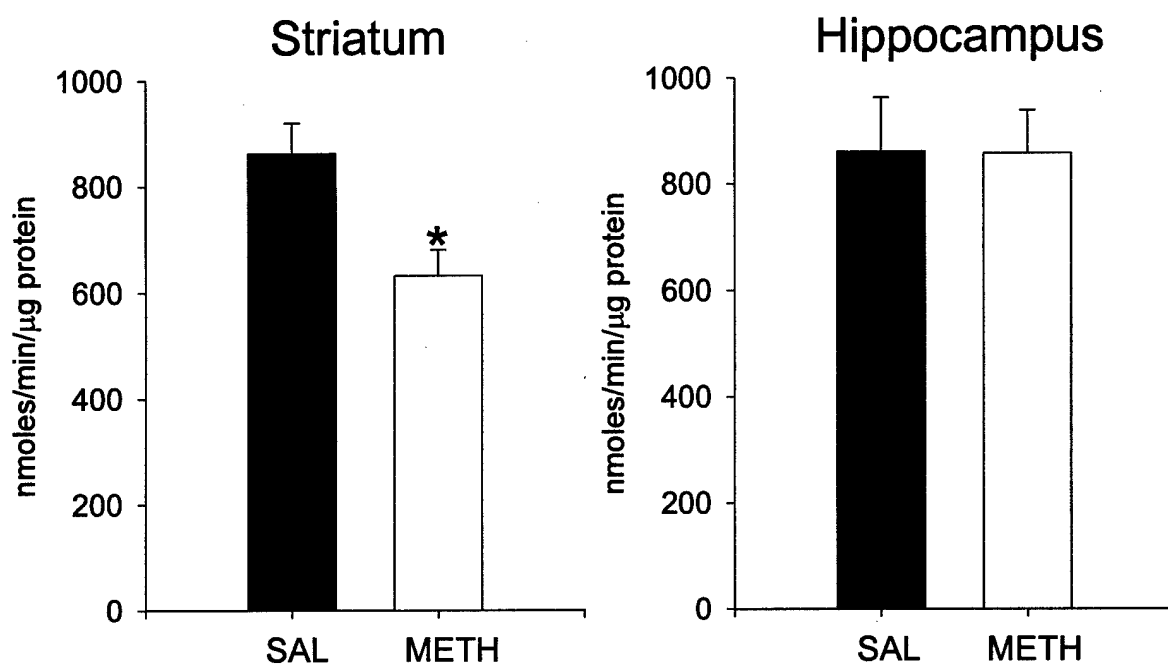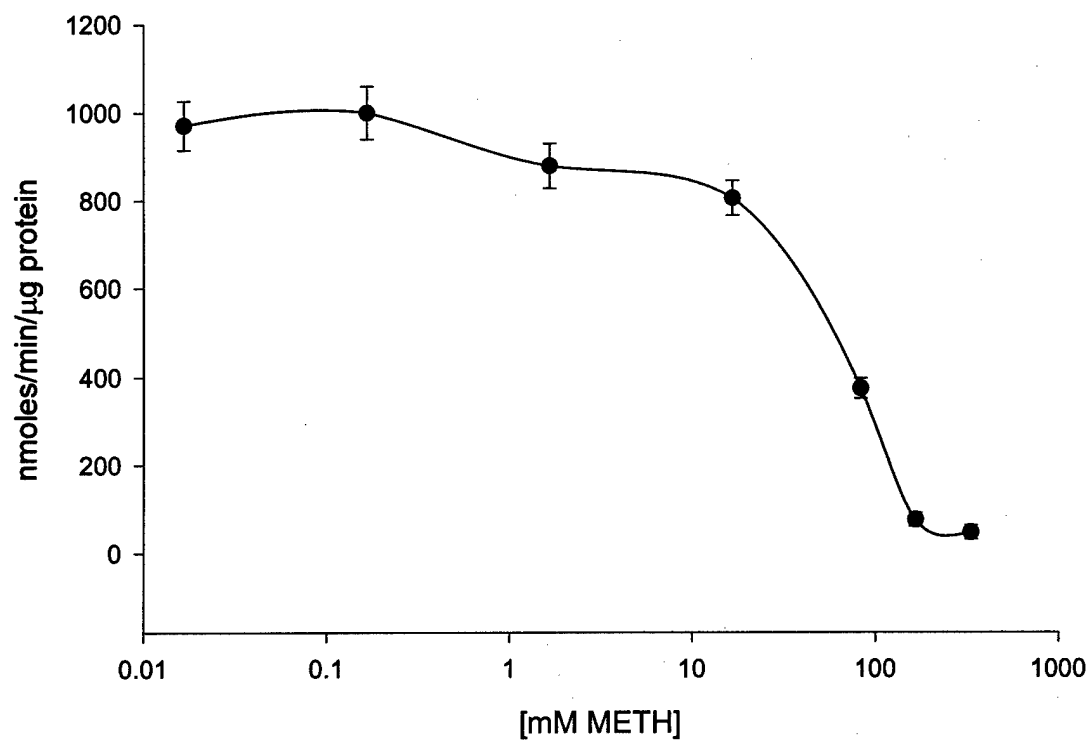Complex I-III

Complex II-III

Figure 2.

Figure 3

Striatum

Hippocampus

Figure 4

Figure 5

Figure 6

Figure 7

SAL   MK-801

600 —

500 —

400 —

300 —

200 —

100 —

0 —

nmoles/min/μg protein

SAL   METH   SAL   METH

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